國 立 交 通 大 學

生化工程所
碩士論文

探討 **Mst3** 在 **HeLa** 細胞誘導細胞凋亡之分子機制

Molecular Mechanism study of Mst3-induced
Apoptosis in HeLa cells

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A thesis

Submitted to Institute of Biochemical Engineering

National Chiao Tung University

In Partial Fulfillment of Requirement

For the Degree of

Master of Science

In

Biochemical Engineering

July 2004

Hsinchu, Taiwan, Republic of China

中華民國九十三年七月

Mst3 誘導細胞凋亡機制之探討

摘要

Mst3 為 52KDa 蛋白質，隸屬人類 **STE20** 相似蛋白激酵素 **(human Ste20 like protein kinase)**家族中的一員，在細胞凋亡過程中扮演重要角色。它具有 N 端的激酵素功能區域及 C 端的調控功能區域，之前研究指出 Mst3 一般存在於細胞質且會被 caspase 活化，且大量表現 Mst3 野生株及截斷株，皆可誘發細胞凋亡之產生。

實驗室便利用廣泛性 caspase 抑制劑 (Z-VAD-fmk) 處理細胞，結果我們發現 Mst3 所導致的細胞凋亡並不會被抑制，而且 Mst3 與 caspase-3 共同表現也不會促進細胞凋現象產生亡，因而推測 Mst3 引起的細胞凋亡或許並非透過 caspase 所誘發或者兩者機制是並行存在的。

另一造成細胞凋亡之訊息傳遞途徑則是透過粒腺體途徑 (mitochondrial pathway)，之前研究指出，當細胞遭遇細胞凋亡時，粒腺體內的膜電位 (membrane potential) 會改變、滲透壓及鈣離子濃度亦會提高，進而導致部份粒腺體凋亡因子，如 cytochrome C、AIF 游離出來。而這些凋亡因子則會受到一些粒腺體蛋白調控，我們研究以免疫染色發現 Cytochrome C 是會與 Mst3 重疊，但 AIF 及 Endo G 則無此現象發生。而 Bcl-2 蛋白質會於 Mst3 存在時，細胞質中的量也會相對減少。我們更意外發現：Mst3 不僅存在於細胞質中，也可在粒腺體中發現。故便需進一步以釐清 Mst3 誘發的凋亡現象中，粒腺體所扮演的角色。

Molecular Mechanism Studies of Mst3-induced Apoptosis in HeLa cell

Abstract

Mst3, a novel human Ste20-like serine/threonine protein kinase, plays an important role in the process of cell apoptosis. It contains a kinase domain at its N-terminus and a regulatory domain at the C-terminus.   Pervious studies have shown that overexpression of Mst3 or its truncated mutant induced apoptosis of HeLa and HEK293 cells. Further studies showed that the Mst3-indued apoptosis did not involve in any known MAPK cascades. Caspases are the central executioner of most classical apoptosis pathway, but its role in Mst3-induced apoptosis of HeLa cells is controversial. The result from this work showed that the universal caspase inhibitor, Z-VAD-fmk, could not supposed Mst3-induced HeLa cell apoptosis suggesting a caspase-independent pathway might exist. Mitochondria play an essential role in trigging caspase-dependent or independent apoptotic process. Many apoptotic factors are present in the mitochondrial intermembrane space. Once activated by apoptosis stimulus, these apoptotic factors may translocate from mitochondria to the cytoplasm or nucleus. The translocation of these apoptotic factors can be regulated by the Bcl-2 family proteins. Our results demonstrated that Mst3 is co-localized with cytochrome C in the mitochondria. This observation indicates that Mst3 may induce apoptosis by altering the cellular localization of cytochrome C. Interestingly the protein level of Bcl-2 was also reduced with the overexpression of Mst3. The subcellular redistribution of other apoptotic factors in response to the overexpression of Mst3 is unknown. Subsequent studies, we found that staurosporine may act differently to induce nuclear translocation of Mst3 as well as AIF proteins. Bcl-2 in nucleus was also increased with the stimulation of staurosporine. The role of Mst3 in mitochondria is unknown so far. The existence of Mst3 in mitochondria may provide a new aspect about the physiological roles of Mst3.

Content

Abbreviations

BSA	Bovine- serum albumin
Caspase	cystein aspartate-specific proteases
DAB	3,3'Daminobenzidine
DMSO	Dimethyl sulfate
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
DIABLO	direct IAP binding protein with low pI
ER	endoplasmic reticulum
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethlyene glycol-bis-(β-aminoethylether) N, N-tetra acetic acid
GFP	Green fluorescent protein
GCK	Germinal center kinase
Mst	Mammalian Ste20-like protein kinaes
PTP	permeability transition pore
PAGE	polyacryamide gel electrophoresis
PBS	phosphate-buffered saline
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidine difluroide
SDS	sodium dodecyl sulphate
Smac	second mitochondrial activator of caspases
TNF	tumor necrosis factor
TdT	Terminal deoxynucleotidyl transferease
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol

TUNEL   **TdT-Utp Nick End Labeling**

z-VAD-fmk   **z-Val-Ala-Asp-fluormethylketone**

Introduction

Apoptosis (or programmed cell death) is a controlled type of cell death that can be induced by a variety of physiologic and pharmacological agents (Kerr et al., 1972). It is an essential physiological process that plays a critical role in controlling the number of cells in embryonic development, differentiation, tissue homeostasis (Hengartner, 2000), and defense against pathogens (Zimmermann et al., 2001). The dysregulation of the apoptotic cell death may be involved in the pathogenesis of a variety of human diseases, including cancers, autoimmune diseases, neurodegenerative disorders and viral infections. The cell undergoing apoptosis exhibits cellular shrinkage, nucleus condensation, DNA fragmentation and cytoplasm vacuolization.

The center of the apoptotic executive machinery is a family of cysteinyl aspartate-specific proteases, termed caspases (Nicholson and Thornberry, 1997). Thus far, the caspase gene family contains 14 mammalian members, which have an unusual and absolute requirement for cleavage at the bond right after aspartic acid (Asp) residues (Stennicke and Deveraux, 1998). Caspases can be divided in two groups according to their roles in apoptosis, the apoptotic initiators (caspase 8, 9 and 10), functioning to activate downstream effector caspases, and the apoptotic effectors (caspase 3, 6 and 7) (Ashkenazi and Cixit, 1998). Effector caspases are responsible for dismantling many cellular components, including proteins important for structure maintainance, cell survival, and for chromatin and nucleus integrity. (Thornbery et al., 1998, Blatt and Glick, 2001). For example, the cleavage of the I^{CAD} (inhibitor of caspase-activated deoxyribonuclease) by caspase 3 is responsible for DNA fragmentation during cell apoptosis (Enari et al., 1998). Caspases also induce the disruption of cell structure by cleaving several

cytoskeletal proteins and their regulatory proteins (Kothakota et al., 1997), focal ashesion kinase (FAK) (Wen. 1997) and p21-activated protein kinase 2 (PAK2) (Rudel and Bokoch, 1997). Notably the activity of caspases are regulated by the Bcl-2 family proteins. The Bcl-2 family proteins can be classified into two sub-groups based on their roles in apoptosis. The anti-apoptotic Bcl-2-like proteins, including Bcl-2, Bcl-x_L, Bcl-x_S, Ced-9, Bcl-w, and Mcl-1, inhibit the apoptosis by preventing cytochrome C from exiting mitochondria and revoking the apoptosis. The pro-apoptotic Bcl-2-like proteins, such as Bcl-2 homology (BH) 1-3, Bax like proteins, Bak and Bok (consisting only the BH3 region) and Bad like proteins, Bid, Bik, Bim and Hrk, induce the apoptotic cell death. The Bax and Bax-like proteins, promote of cytochrome C from mitochondria which activates the caspase-9-dependent apoptosis cascade. Notably the initiation of apoptosis depends on the ratio of Bcl-2 and Bax proteins in the cells. The predominance of pro-survival Bcl-2 proteins over pro-apoptotic Bcl-2-like proteins in the cells may block the initiation of apoptosis, whereas the predominance of the Bax protein in the cytoplasm promotes the implementation of death signals.

AIF has a mitochondrial localization signal at its N-terminal and is confined to the mitochondria in the absence of apoptotic signal. Once imported into the mitochondrial intermembrane space, the mitochondrial localization signal of AIF is cleaved off to generate a mature 57 kDa AIF (Wu et al., 2002). Mature AIF translocates to the nucleus in response to apoptotic stimuli and causes DNA fragmentation (Susin et al., 1999). Endonuclease G, similar to AIF, can translocate to the nucleus and facilitate the DNA fragmentation (Li et al., 2001). Another mitochondrial factor, Smac (also called DIABLO), involves in apoptotic process by inactivating the inhibitor of apoptosis (IAP) (Mackenzie et al., 2000). All of these factors induce apoptotic cell death in response to apoptotic stimuli

through a caspase-independent pathway as implicated by the observation that the apoptosis induced by these factors cannot be suppressed by the pan-caspase inhibitor Z-VAD-fmk (Wu, 2002; Penninger and Kroemer, 2003).

The cell death receptors, including TNF-α receptor (TNF-R) and Fas-ligands receptor Fas/APO-1/CD95 conduct apoptotic signals by converting pro-caspase 8 to the active one, which in turn activates Bcl and Bax. The activation of DEDs, DED1 and DED2, trigger a signaling cascade that modify and activate proteases ICE/CED-3. The first step is the conversion of the non active pro-caspase-8 into the active caspase-8. The caspase-8 then activates caspase-3 and Bid. The release of cytochrome C from mitochondria can be induced by the formation of channel-like structure of activated Bid and Bax complex on the surface of mitochondrial outer membrane. Cytochrome C then complexes with Apaf-1 and recruits caspase-9, forming the apoptosome (Zou H et al., 1997; Li P et al., 1997). The activated caspase-9 in turn actives caspases 3, 6, and 7 induce cell apoptosis. (Ravagnan et al., 2002).

The endoplasmic reticulum (ER) is an essential organelle that regulates protein synthesis, lipid synthesis, cellular responses to stress, and calcium (Ca^{2+}) storage (Berridge, 1995; Rapport, 1992). A variety of stimuli, including Ca^{2+} ionophore (A23187), inhibitor of glycosylation, chemical toxicants and oxidative stress, can induce stress on ER and ultimately lead to cell death (Liu et al., 1998; McCormick et al., 1997). Caspase 12 is thought to associate with ER (Nakagawa et al., 2000) and involve in the apoptosis-induction by the stress on ER (Nakagawa et al., 2000; Yoneda et al., 2001). The mechanism underlying caspase 12 activation is unclear so far. However, two possible mechanisms are proposed. One mechanism may involve the binding of caspase 7 and caspase 12 to GRP78 on ER surface resulting in autoactivation of csapase 12 (Yoneda et al., 2001). Alternatively, the

activation of casapse 12 may be mediated by calpain (Nakagawa et al., 2000). Apoptosis can be induced in several ways, some clearly involving Ca^{2+}. This, coupled to the accepted role of proteolysis in the degradation phenomena associated with the death of cells has led to frequent suggestions of possible role for calpains. More significantly, dexamethasone was found to result in calpain activation, as determined by the increase in Ca^{2+}–dependent proteolysis of added substract and, especially, by the disappearance of the 80 kDa form of calpain in favour of the activated autolytic products (Ernesto and Maurizio, 1998). Msts (mammalian Ste20-like protein kinase) are human counter parts of the budding yeast Ste20 protein kinase that involve in the pheromone response. Notably, Ste20 serves as a link between the G protein and MAPK cascade (Ramer et al., 1993). At least 30 members of mammalian Ste20-like protein kinases have been identified and cloned. They can be divided into two subfamilies based on the locations of their kinase domains: the p21-activated kinase (PAK) subfamily and germinal center kinase (GCK) subfamily. The PAKs consist of a C-terminal kinase domain and an N-terminal regulatory domain, with a small GTPase Rac1/Cdc42 binding region. GCKs possess an N-terminal kinase domain and a C-terminal regulatory domain with unknown function. Several lines of evidence have shown that some Ste20-like protein kinases function as potential upstream activations of MAPK pathways (Shi et al., 2000). Other than this ability to induce cell proliferation, mitogen-activated protein kinases, including extracellular signal-related kinase (ERK1/2), JNK/SAPK and p38 MAPK, were implicated to play role in the progression of apoptosis. (Kyriakis et al., 1996; Fanger et al., 1997).

Four Mst family members have been identified, including Mst1/Krs2 (56 kDa),

Mst2/Krs1 (56.5 kDa), Mst3 (47.5 kDa) and Mst4/MASK (46 kDa). They can be classified into two GCK subfamilies, GCKII (Mst1 and Mst2) and GCKIII (Mst3 and Mst4), based on the sequence homology (Dab et al., 2001). Mst1, Mst2 and Mst3 have been shown to be involved in the process of cell apoptosis, while the role of Mst4 is controversial (Lin et al., 2001). Mst1 and Mst2 can be activated by apoptotic agents, including staurosporine, Fas-ligand, okadaic acid, synthetic apoptotic peptide MT-21, anti-cancer drugs and environmental stresses, such as heat shock and high concentrations of sodium arsenite (Graves et al., 1998; Reszka et al., 1999). With the stimulation of apoptotic signals, Mst1 can be prototypically activated by caspases, which can be specifically blocked by the selective caspase inhibitor (Graves et al., 1998; Lee et al., 2001). Upon activation Mst1 may transmit the apoptotic signal through either MEKK1-based MAPK pathway (Graves et al., 2001) or the JNK/SAPK pathway. Mst4, a member of GCKIII subfamily, was found to facilitate cell growth and transformation via MEK/ERK pathway (Lin et al., 2001). However, recent studies in our laboratory have shown that Mst4 may also play a role in triggering the apoptosis of HEK293 and MCF-7 cells (Chen, P. H. 2003).

Mst3, a novel human Ste20-like serine/threonine protein kinase, shares 88% sequence similarity to the kinase domain of SOK1 and Mst4. Pervious studies have shown that overexpression of full-length or truncated Mst3 induced apoptosis of HeLa and HEK293 cells. Subsequently, a caspase-cleavage site in Mst3 was identified at AETD[313] (Huang et al., 2002). After truncation, Mst3 translocates from cytoplasm to nucleus. Further works were carried out to elucidate that Mst3 contained NLS (nucleus localization signal) and NES (nucleus exporting sequence) signals at the C-terminus of its kinase domain (278-292) and in the C-terminal regulatory domain, respectively (Huang et al., 2002; Wang et al., 2004).

Furthermore Mst3 does not activate any known MAPK pathway (Schinkmann et al., 1997; Huang et al., 2002).

Although the molecular mechanism underlying the Mst3-induced cell apoptosis is unclear so far, caspases were shown to play a role in activating Mst3 in Jurkat cells (Huang et al., 2000). Caspases 3, 7 and 8 were suggested to activate Mst3 by removing it C-terminal regulatory domain. The cleavage of the C-terminal regulatory domain triggered the nuclear translocation of truncated $Mst3^{WT\triangle314}$. Hence, it will be interesting to understand the roles of caspases in Mst3-induced cell apoptosis and nucleocytoplasmic translocation of Mst3.

Apoptosis can be initiated by disrupting the integrity of mitochondria, activating the cell surface death receptor and by endoplasmic reticulum (ER) stress-mediated cell death (Rammohan, 2001). Mitochondria play an important role in trigging apoptotic process. At the onset of apoptosis, the mitochondrial membrane potential is altered and the apoptotic factors relaesed in the intermembrane space. The factors include cytochrome C, apoptosis inducing factor (AIF), endonuclease G (EndoG) and Smac/DIABO.

Since mitochondria play a central role in the pathways of caspase-dependent and caspase-independent cell apoptosis. We like to find out the role of mitochondria and the mitochondrial apoptotic factors in the Mst3-induced cell apoptosis.

Materials and Methods

Cell Line and Cell Culture

HeLa (Human cervical cancer cell line) cell line was purchased from Food Industrial Research and Development Institute, Hsin-Chu, Taiwan. HeLa cells were grown in the DMEM medium containing 10% FBS and 1% each of penicillin and streptomycin. Cells were incubated at 37 ℃ in a humidified incubation chamber containing 95% air and 5% CO_2. Cells were sub-cultured every 2-3 days.

Chemicals and Reagents

Caspase inhibitor (z-vad-fmk) was the product of Merck; Inhibitors for p38-MAPK (SB220025) and a PD98059 inhibitor for Jun-N-terminal kinase (JNK), were purchased from Biosource. The TdT-FragEL DNA Fragmentation Detection Kit was obtained from Oncogene. Specific antibody for Bax was purchased from Biosource. Antibody for Bcl2 was obtained from Serotec. Antibody for AIF was brought from Santa Cruz. The antibody for cytochrome c oxidase subunit IV (COXIV) was purchased from Molecular Probes. The antibodies for Bcl-XL, Bad, Bid and EndoG and Cytochrome C Releasing Apoptosis Assay Kit were brought from BioVision, Rhodamine (TRITC) was purchased from Santa Cruz. MitoTracker Red dye was purchased from Molecular Probes. Fetal bovine serum (FBS) and Dulbecco's modified egale medium (DMEM) were purchased Hyclone. The trypsin-EDTA, penicillin and streptomycin and lipofectamine reagent were brought from GIBCO BRL. G418 was product of Calbiochem. NP-40 was pruchased from PIERCE. Other chemicals and reagents used were reagent grade.

Exogenous β- galactosidase Activity Assay

For the exogenous β-galactosidase Activity assay, HeLa cells were cultured in the 35-mm culture dishes to about 70% confluence. The expression plasmid pcDNA 3/Mst3^{WT} or pcDNA 3/Mst3^{KR} (0.7 μg each) was then co-transfected with pSV-Laz vector (0.7 μg) into the cells by using lipofectamine (GIBCO). Twenty-four hours following transfection, cells were lysed by incubating with 200 μL cell lysis buffer (20 mM PIPES, PH 7.2, 100 mM NaCl, 1 mM EDTA, 0.1% CHAPS and 10% sucrose) at room temperature for 15 min. The β-galactosidase activity assay was carried out by mixing 100 μL cell lysate with 200 μL PM2 buffer (0.1 M Na_3PO_4, 1 mM $MgSO_4$, 0.2 mM $MnSO_4$), and 200 μL O-nitrophenyl-β-D-galactopyranoside (ONPG; 4 mM/mL). Reaction mixture was then incubated at 37   for 6 h or until a yellow color developed. Stop the reaction by adding 0.5 mL 1M Tris-HCl, PH 7.0. The activity of β-galactosidase of each sample was determined by meaning the absorbance at 420nm.

TUNEL (TdT-Utp Nick End Labeling) Assay

DNA fragmentation induced by Mst3 was determined using TdT-FragEL kit (Oncogene) according to manufacture's instruction. HeLa cells were cultured on the cover-glass slide in a 35-mm cultural dish to about 70% confluence before subjecting to transient transfection and transfected with pcDNA3-Mst3 or its mutants. After recovering for 16 h, cells were washed twice with TBS buffer (20 mM Tris-HCl, PH 7.6, 140 mM NaCl) and fixed with 4% formaldehyde in 1× TBS at 4 ℃ for 30 min. After fixation, cells were rinsed once with TBS and permeabilized by adding 2 mL TBST solution (20 mM Tris-HCl, PH 7.6, 140 mM NaCl and 0.05% Tween 20) on the top of cells at room temperature for 5 min. After

permeabilization, 100 μL 3% H_2O_2/methanol were added and incubated at room temperature for 5 min to deprive the endogenous peroxidase. Cells were washed twice with TBS and incubated with 100 μL 1× TdT equilibration Buffer (1M sodium cacodylate, 0.15M Tris, 1.5 mg/ml BSA, 3.75 mM $CoCl_2$, pH 6.6) and incubated at room temperature for 20 min. Following incubation, carefully remove the TdT equilibration buffer from the glass slide, and immediately applied 60 μL TdT- labeling reaction mixture (3 vials containing a mixture of labeled and unlabeled deoxynucleotides at a ratio optimized for DNA Fragment End Labeling with TdT) onto the cells. The labeling reaction was performed by placing the slide in a 37 ℃ incubator for 90 min. After incubation, the reaction was terminated by adding 100 μL stop buffer (0.5 M EDTA, pH 8.0) at room temperature for 5 min. Rinse the entire specimen once with TBS and incubate with 100 μL blocking buffer (4 % BSA in PBS) at room temperature for 10 min. Remove the blocking buffer, immediately add 100 μL of 1× peroxidase-conjugated streptavidin solution to the cells at room temperature for 30 min. The HRP activity was then detected by incubating 100 μL DAB solution at room temperature for 10 min. After reaction, cells were used with demonized H_2O once, followed by adding 100 μL methyl green (0.3 % methyl green) counter stain solution at room temperature for 3 min. Cells were then washed with 100% ethanol and dried the specimen under the room temperature. Finally, observe the cells under the light microscopy and count the number of positively stained cells.

Cell Transfection

The plasmid transfection into cells was performed using lipofectamine reagent (GIBCO). Briefly, HeLa cells ($2.5×10^5$/well) were grown in a 6-well culture plate

at 37   to about 70% confluence. The transfection mixture was freshly prepared by mixing solution I (1 μg DNA in 100 μL Opti-MEM) solution II (4 μL lipofectamine in 100 μL Opti-MEM). The resulting mixture was then incubated at room temperature for 30-45 min. After incubation, add 800 μL Opti-MEM into the transfection mixture and mix well. Remove the old culture medium from each well and smoothly lay 1 mL transfection mixture on the top of the cells. The incubation was carried out at 37   for 5 hr in a CO_2 incubator. Following incubation, replace the transfection mixture with 2 mL fresh DMEM containing 10% FBS. The cells were then recovered at 37   for about 14 h, before the subsequent treatments. The translocation of mitochondrial proteins and were performed, using immunofluorescent staining. The z-VAD-fmk (50 μM), JNK inhibitor (20 μM) and ERK inhibitor were used to treat the cells. The treatment was processed at 37 incubator for 24 hr.

Immunofluorescent Staining

The translocation of various mitochondrial proteins in cells was monitored by immunofluorescent staining HeLa cells (2.5×10^5/well) were cultured in a 35-mm culture dish containing a piece of 2×2cm cover-glass to about 70-80% confluence. Cells were then transfected with pEGFP-Mst3^{WT} (1 μg) or pEGFP-Mst3^{KR} (1 μg). After transfection, Cells were recovered in the DMEM containing 10% FBS for 14 hours. The cells on the cover slip were washed once with 1× PBS and fixed with 4% paraformaldehy in PBS containing 50mM $NaHCO_3$ at room temperature for 20 min. Cells were then blocked by 5% FBS at room temperature for 1 h, followed by incubating with first antibody (1: 200 dilution for AIF antibody, 1:100 dilution for EndoG and cytochrome C antibodies) at room temperature for 1-3 h in a humidified chamber. After incubation, cells were washed three times with PBST

(20 mM phosphate, PH 7.6, 140 mM NaCl and 0.05 % Tween 20) followed by incubating with the secondary antibody (1:200 dilution for Rhodamine-conjugated secondary antibody and 1:400 dilution for FITC-conjugated secondary antibody) at room temperature for 30 min. After incubation, cells were the nucleus of further stained with 200 ng/mL 4'6'-diamidino-2-phenylindole (DAPI) in the dark, at room temperature for 15-30 min. After staining, cells were washed three times with PBST. The fluorescent images were acquired by the confocal laser scanning microscope (FV500, OLYMPUS).

Isolation of Cytoplasmic and Nuclear fractions

HeLa cells were cultured in a 100-mm cultural dish to about 70-80% confluence. Cells were then harvested by scrapping and collected via centrifugation. Cells were re-suspended in 200 μL buffer I (10 mM Tris-HCl, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA and 1 mM DTT) and incubated on ice for 15 min. After incubation, 10% NP40 (12.5 μL) was added into the cell suspension and vigorously vortexed for 10 second under the room temperature. Centrifuge the tubes at 13200 rpm for 30 second to collect the nuclei. Transfer the upper cytoplasmic fraction to a new 1.5 mL microfuge tube. To the nuclei pellet buffer II (20 mM Tris-HCl, pH 7.9, 40 mM NaCl, 1 mM EDTA, 1 mM EGTA and 1 mM DTT) were added, followed by a vigorous shaking in the cold for 15 min. After incubation, mixture was centrifuged at 13200 rpm in the cold for 30 second. Transfer the upper nuclear fraction to a new 1.5 mL tube and stored at −80   or subjected to experiment directly.

Isolation of Mitochondrial proteins

The isolation of mitochondrial proteins was performed using Cytochrome C

releasing Apoptosis Assay kit following manufacture's protocol. Briefly, cells (5×10^7) were collected by scrapping in 1 mL PBS, followed by centrifugation at 1400 rpm for 5 min at 4 ℃. The pellet of cells were re-suspended 1 mL 1× Cytosol Extraction buffer mix containing 1mM DTT and protease inhibitor and incubated on ice for 10 min. Transfer homogenate to a 1.5 mL microfuge tube and centrifuge at 2000 rpm at 4 ℃ for 10 min. Transfer supernatant to a 1.5 mL microfuge tube and centrifuge at 13200 rpm at 4 ℃ for 30 min. Collect the supernatant as cytoplasmic fraction. Re-suspend the pellet in 100 μL of 1× Mitochondria Extraction Buffer containing DTT and protease inhibitor. Vortex for 10 second and save as mitochondria proteins.

Western Blotting

HeLa cells or pcDNA3.0 and pcDNA/Mst3^{KR} stably transfected cells were cultured in a 100-mm cultural dish to about 70-80% confluence. The cell lysate was prepared by incubating cells with cell lysis buffer (50 mM Tris pH 7.4, 1 mM NaCl, 1 mM EGTA, 1mM EDTA, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/ml Aprotinin and 10 μg/ml Leupeptin) on ice for 30 min. Total protein (60 μg) of each cell lysate was loaded onto a 12% SDS-polyacrylamide electrophoresis gel (SDS-PAGE). After electrophoresis at 150 Volt for 1.5 h, the separated protein bands were transferred to a polyvinylidine difluroide (PVDF) membrane on a BIO-LAB Mini-Trans Blot Electrophoretic Transfer Cell soaked in a transfer buffer (12.5 mM Tris, 96 mM glycin, 20% methanl and 0.1% SDS). After transblotting, PVDF membrane was blocked with 5% skim milk in PBST at room temperature for 1-2 h. The membrane was then incubated with first antibody (1: 5000 dilution for β-actin, 1: 1000 dilution for

Mst3 antibody, 1: 2000 dilution for AIF antibody, 1: 500 dilution for cytochrome C antibody, 1: 500 dilution for EndoG antibody, 1:1000 dilution for Bcl2 antibody, 1:1000 dilution for Bax antibody and 1: 500 dilution for Bcl-X$_L$) at room temperature for 1h or at 4 for overnight. After incubation, the membrane was washed three times with PBST, followed by incubating with secondary antibody (1: 1000 dilution for host rabbit, mouse and goat) at room temperature for 1 h. After washing three times with PBST and once with PBS, 1 mL ECL was spread on the PVDF membrane for chemiluminescent reaction. The specific protein bands were visualized by exposing on a X-ray film (Hyperfilm). Protein concentration for SDS-PAGE was determined by Braford reagent (Bio-Rad).

Results

Mst3 Induced Apoptosis of HeLa cells

It has been demonstrated that Mst3 could induce the major characteristics apoptosis in HeLa cells (Huang *et al.*, 2002). Overexpression of Mst3 induced DNA fragmentation, a major characteristic of cell apoptosis of HeLa cells. The TdT-FragEL KIT was further used to determine whether Mst3-induced DNA fragmentation in HeLa cells. The Terminal deoxynucleotidyl transferase (TdT) catalyzes the labeling of exposed 3'-OH ends due to DNA fragmentation with biotin-conjugated nucleotide. The HRP-conjugated streptavidin was then used to recognize the biotin- conjugated newly exposed 3'-end. Diaminobenzidine (DAB) reacts with the labeled sample to generate the insoluble dye deposit (brownish red) at the site of DNA fragmentation. Counterstaining of cells with methyl green could aid in the morphological evaluation and characterization of normal and apoptotic cells. The result shows that DNA fragmentation could be induced in HeLa cells by transiently transfected either with Mst3^{WT} (Fig. 1D) or truncated Mst3$^{\triangle 314}$ (Fig. 1E). In consist, HeLa cells either untreated, or transfected with vector alone or Mst3^{KR} showed no sign of DNA fragmentation (Figs. 1A, 1B and 1C).

Mst3 Induces a Caspase-independent Apotosis

Caspases are the central executioners of most classical apoptotic pathways and are suggested to involve in Mst3-mediated apoptosis in Jurkat cells (Huang *et al.*, 2002). We want to examine whether Mst3-induced HeLa cell apoptosis of HeLa cells is a caspase–dependent process. Accordingly, an exogenous β-galactosidase assay was performed by co-transfecting pSV-LacZ plasmid with Mst3^{WT} or its muntants into the HeLa cells. Six hours after transfection, a universal-caspase

inhibitor zVAD-fmk (50 μM) was added into in the culture medium. Notably, Mst3^{WT}-induced cell death could not be inhibited by 50 μM zVAD-fmk (Fig. 2). Both pcDNA3.0 vector and Mst3^{KR} showed no significant effect on HeLa cells and were not affected by caspase inhibitor. The result suggests that Mst3 may induce apoptosis of HeLa cells in a caspase-independent manner.

Effect of JNK nor p38/MAPK inhibitor in Mst3-induced apoptosis

Mst1 or Mst2 has been shown to induce apoptosis through the JNK/SAPK and p38/MAPK pathways (Lin et al., 2001). Early works in our laboratory also indicated that Mst4-induced apoptosis was a JNK-dependent process. Although Mst3 was shown to induce cell apoptosis through a pathway independent of any known MAPK pathway (Huang et al., 2002), we want to further confirm this observation using exogenous β-galactosidase assay in the presence of SP600125, an inhibitor for JNK or SB220025, an inhibitor for p38/MAPK. As expected Mst3-induced apoptosis of HeLa cells was not affected by JNK inhibitor, SP600125 (Fig. 3) and p38/MAPK inhibitor, SB220025 (Fig. 4), confirming the previous observations (Huang et al., 2002).

Localization of endogenous Mst3 in HeLa Cells

Pervious study reveals that full length Mst3 was mainly present in the cytoplasm, whereas Mst3^{WTΔ314} accumalceted in the nucleus. The nucleus localization of Mst3^{WTΔ314} was suggested to play a role in promoting apoptosis of cells. However, this result can not explain the observation that overexpression of full-length Mst3^{WT} can also induce apoptosis (Huang et al., 2002). Interestingly, Mst4, a closely related isoform of Mst3, was shown to distribute at Golgi apparatus, especially at cis-Golgi network. This observation promotes us to check the

subcellular distribution of Mst3 in HeLa cells by western blotting. The cytoplasmic, nuclear and mitochondrial fractions were first prepared as indicated in Materials and Methods. The existence of endogenous Mst3 in various fractions was determined by Western blotting. To our surprise, we found that endogenous Mst3 was not just only present in the cytoplasm but also existed in the mitochondria (Fig. 5). Consistent with previous observation, only little Mst3 is present in the nucleus. This observation was further demonstrated by monitoring the fluorescent images of mitochondria, mitochondrial marker (Molecular Probe) in EGFP-Mst3^{WT} and EGFP-Mst3^{KR} transiently transfected HeLa cells. Fig. 6 shows that EGFP-Mst3 ^{WT} or EGFP-Mst3^{KR} (green) co-localizes with the mitotracker (red), indicating that Mst3 is present in mitochondria. The reason for the presence of Mst3 in mitochondria is, however, remain elucidated.

Locatization of apoptotic factors in HeLa Cells

Several lines of evidence have shown that many apoptotic factors, including cytochrome C, apoptosis inducing factor (AIF) and endonuclease G (Endo G), are present in the mitochondria. Cytochrome C was in the intermembrane space (IMS) (Grup et al., 2003; Yu et al., 2002; Ohsato et al., 2002). EndoG and AIF were also confined to the mitochondrion but the exact location is not clear so far. Thus, it will be very interesting to know if any one of these apoptotic factors can associate with Mst3, which is just indicated to exist in mitochondria. The existence of apoptotic factors in mitochondria was first demonstrated by double immunofluorescent staining. Mitochondria were stained with a specific fluorescent conjugated mitochondrial marker, mitotracker red (Molecular Probe). Fluorencent images of apoptotic factor and mitotracker were observed under a confocal laser scanning microscope (Fig. 7). The result clearly shown that the apoptotic factor proteins

studied in this work are resided in the mitochondria. We next try to find out whether Mst3 associates with any of the above apoptotic factors. HeLa cells were transiently tranfected with EGFP-Mst3^{WT} or EGFP-Mst3^{KR} plasmid prior to the immunofluorescent staining of apoptotic factors. Sixteen hours after transfection, cells were fixed and detected by the antibody specifically against one of above apoptotic factors. The localization of apoptotic factors was visualized using Rhodamine-conjugated secondary antibody. Interestingly, we found that the fluorescent images of cytochrome C and EGFP-Mst3 and EGFP-Mst3^{KR} were colocalize (Fig. 8), presumably in the mitochondria. The fluorescent image of AIF was also observed to overlap with that of EGFP-Mst3^{KR} (Fig. 9). EndoG, on the contrary, did not show any sign of overlapping with EGFP-Mst3 (Fig. 10).

Effect of exogenously expressed Mst3^{WT} and Mst3^{KR} on the Bcl-2 protein level

Members of Bcl-2 family play important roles in the regulation of cell apoptosis. These proteins can be classified into two subfamilies based on their role in the regulation of apoptosis. The anti-apoptotic Bcl-2 subfamily, including Bcl-2, Bcl-X$_L$ and Bcl-W, promotes cell survival; the pro-apoptotic subfamily, including, Bax, Bid and Bak, induces cell death. Among Bcl-2 homologs, Bcl-2, Bcl-X$_L$ and Bax represent the most well characterized members. Bcl-2 can promote cell survival against various cytotoxic insults (Biose et al., 1995) and Bcl-X$_L$ could inhibit apoptosis induced by free radical, UV light and chemicals. Bax, on the other hand, can cause the release of cytochrome C from mitochondria and activates caspase 3-dependent cell death. Hence, it is worth to know the influence of Mst3 in the expression of Bcl-2-like proteins in HeLa cells. HeLa cells transfected with pcDNA-Mst3^{WT} and pcDNA-Mst3^{KR} were used as the models to find out the effects of Mst3^{WT} and Mst3^{KR} to the protein level of Bcl-2, Bcl-X$_L$, and Bax. The

result reveals that the protein level of Bax and Bcl-X$_L$ was unchanged. The protein level of Bcl-2 was reduced with the overexpression of Mst3^{WT} (Fig. 11). In the presence of Mst3^{KR}, however, Bcl-2 level was almost unchanged. This result suggests that Mst3-induced cell death may be proceed via changing the availability of anti-apoptotic Bcl-2.

Staurosporine induces nuclear localization of Mst3

Earlier work has shown that the association of Mst3 with cytochrome C may involve the regulation of cell apoptosis. Interestingly, the apoptosis induced by some anti-cancer drugs including, staurosporine, etopside and camptothecin involved in the interference of mitochondrial functions. For example, release of cytochrome C can be induced by etoposide (Yang et al., 1997; Sun et al., 1999). Staurosporine can induce the proteolytic cleavage of Mst3 and hence the apoptotic cell death of Jurkat cells (Huang et al., 2002). The effect of staurosporine on Mst3 in HeLa cells, however, has not been studied. The HeLa cells were treated with 0.5 μM staurosporine at 37℃ for 3 h. Subsequently, cells were harvested and fractionated to prepare cytoplasmic, nuclear and mitochondrial fractions. Cells treated with 0.05 % DMSO was used as control. Surprisingly, we found that staurosporine could induce a nuclear translocation of full-length Mst3 in HeLa cells. The subcellular redistribution of Mst3 and other apoptosis regulating factors, such as AIF, EndoG and Bcl-2 was studied. The nuclear localization of AIF also induced by the treatment of 0.5 μM stauroaporine (Fig. 14).The protein level of Bcl-2 in nucleus was also increased with the stimulation of staurosporine.

Discussion

Pervious studies have revealed that overexpression of Mst3 might induce apoptosis of HeLa and HEK293 cells (Huang et al., 2002). To further confirm the role of Mst3 in apoptosis DNA fragmentation, one of the hallmarks of apoptosis, the cell singles were analyzed by TUNEL assay. I found that Mst3 could induce DNA fragmentation in HeLa cells as demonstrated by the brownish staining in nuclei of Mst3^{WT} and Mst3 $^{WT\Delta314}$ transfected cells (Fig 1).

GCK II family protein kinases, such as Mst1 and Mst2, have been shown to activate either JNK/SPAK or p38/MAPK signaling pathway upon overexpression (Dan et al., 2001; Widmann et al., 1999). Mst1, sharing 56% sequence homologous with Mst3, is involved in the JNK and p38 pathways (Taylor et al., 1996). It was also found that Mst4 could induce apoptosis of MCF cells via a JNK-dependent pathway. Notably, Mst3 was suggested to induce an unique signaling pathway without the involvement of any known MAPK cascades. This work further confirmed this observation by finding that Mst3-indued HeLa cell apoptosis was not affected by JNK or p38 inhibitors. Caspases are the central executives of most classical apoptosis pathways. Although caspase3 is shown to cleave and activate various Ste20 group kinases including Mst1, Mst2 or PAK2 (Dan et al., 2001), Mst3-induced apoptosis of HeLa cells can not be inhibited by its inhibitor, Z-VAD-fmk. This result suggests that Mst3 may induce cell death via a caspase-independent pathway in HeLa cells (Daugas et al., 2000).

Mitochondrion is one of the important organelles in cells playing multiple roles to supply energy needed for cellular functions as well as to mediate ageing, oxidative stress and cell apoptosis. Mitochondria mediate cell apoptosis via several ways, including activating caspase activators, changing redox status of the electron

transport chain, disrupting the mitochondria transmembrane potential, and cooperating with anti- and pro-apoptotic Bcl-2 family proteins (Adams and Cory, 1998). Release of cytochorome C from mitochondria induces the activity of caspase 9, and subsequently, activates caspase 3 and following apoptotic processes, termed caspase-dependent pathway. Numerous studies have demonstrated that apoptosis induced by some apoptotic stimuli cannot be inhibited by the pan-caspase inhibitor, Z-VAD-fmk, suggesting the existence of caspase-independent apoptotic pathway (Wu, 2000; Okuno et al., 1998). Normally, theseapoptotic factor proteins are present in the mitochondrial intermembrane space. Once activated by any apoptosis stimulus, these apoptotic factors may translocate to the cytoplasm or the nucleus. Surprisingly, we found that cytochrome C is co-localized with mst3 in the mitochondria. This result indicated that Mst3 might induce apoptosis by altering the cellular localization of cytochrome C. The role of Mst3 in mitochondria is unknown so far. The existence of Mst3 may provide a new physiological aspect about Mst3 and showed the important for its role in regulating cell apoptosis. The translocation of these apoptotic factors are regulated by pro-survival Bcl-2 family members, such as Bcl-2 and BclX$_L$, and pro-apoptotic Bcl-2 family members, such as Bax, Bad and Bim (Adams and Cory, 1998). The ratio of pro-survival and pro-apoptotic Bcl-2 family members determines the susceptibility of cells to a certain apoptotic signal.

This postulation is partially supported by the finding that the protein level of Bcl-2 was reduced with overexpression of Mst3 in HeLa cells. The subcellular distribution of other apoptotic factors in response to the overexpression of Mst3 is unknown. Since Mst3-induced apoptosis is caspase-independent, the distribution of apoptotic factors altered by the activation of Mst3 is expected and will be studied in the future.

Staurosporine has been known to induce the proteolytic activation of Mst3 in Jurkat cells (Huang et al., 2002). In this we found that staurosporine may act differently by inducing the nuclear translocation of Mst3 and AIF. Bcl-2 in nucleus was also increased with the stimulation of staurosporine.

There is mounting evidence shows that some forms of cell death can be mediated by calcium release from ER stores, perhaps accompanied by calcium entry from the extracellular milieu (Baffy et al., 1993; Pu and Chang, 2001; Abeele et al., 2002). This redistribution of calcium leads finally to a decisive induction of Pore Transition (PT) through mitochondrial calcium overload. Other calcium-dependent mechanisms may or may not involve mitochondria, such as calpain activation. The interorganelle flow of calcium is facilitated by direct or indirect connections between mitochondria and the ER. Bcl-2 is known to regulate the redistribution of calcium, particularly reducing the amount of calcium releasable from the ER, thus preventing mitochondrial calcium overload and ensuring cell survival (Abeele et al., 2002). Bax, on the other hand, produce the opposite effect, making more calcium available for inducing PT in mitochondria (Nutt et al., 2002). Although regulation of Bcl-2 family involved many different cell death pathway, the results of this study indicates that Mst3 may play an essential role to regulate the release of caspase activator or other apoptotic factors with the cooperation of Bcl-2 family members.

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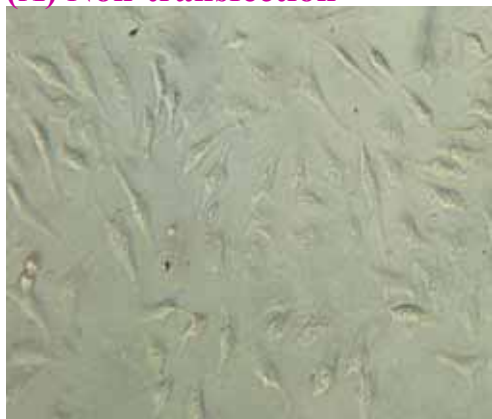
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(A) Non-transfection

(B) pcDNA3.0
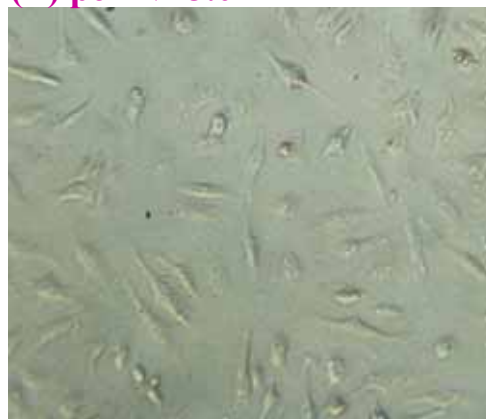
(C) pcDNA/Mst3^{KR}
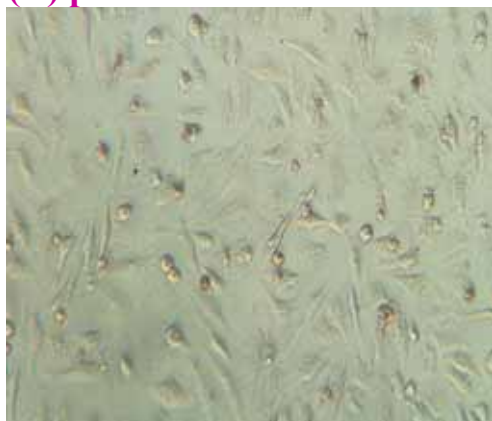
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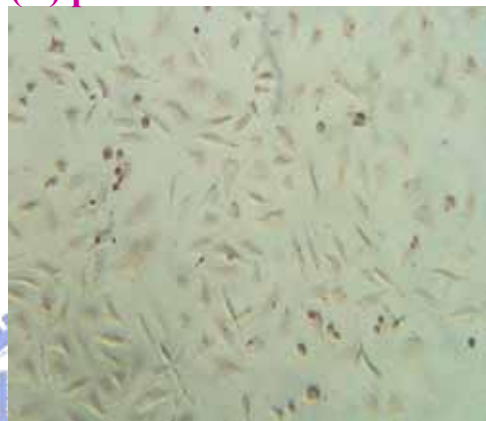
(E) PcDNA/Mst3$^{WT\Delta314}$
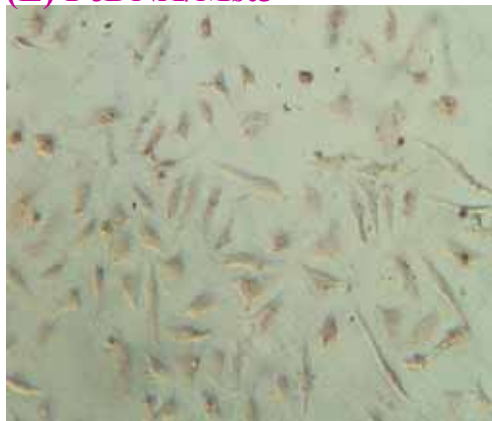
Fig. 1 The TUNEL assay of HeLa cells. HeLa cells were transfected without (A) or with pcDNA3.0, (B) pcDNA/Mst3^{KR}, (C) pcDNA-Mst3^{WT}, (D) and PcDNA/Mst3$^{WT\Delta\,314}$ (E). Cells were recovered for 18 h prior to the TUNEL assay. Cells transfected with pcDNA3.0 and pcDNA/Mst3^{KR} were used as negative controls. Cells underwent DNA fragmentation were stained brownish red in the nucleus, whereas the viable cells exhibited green in the picture.

| Relative activits	pcDNA 3.0	pcDNA+Z-VAD-fmk	Mst3WT	Mst3WT+Z-VAD-fmk	Mst3KR	Mst3KR+Z-VAD-fmk
Relative activits	1	1	0.49	0.47	0.88	0.93
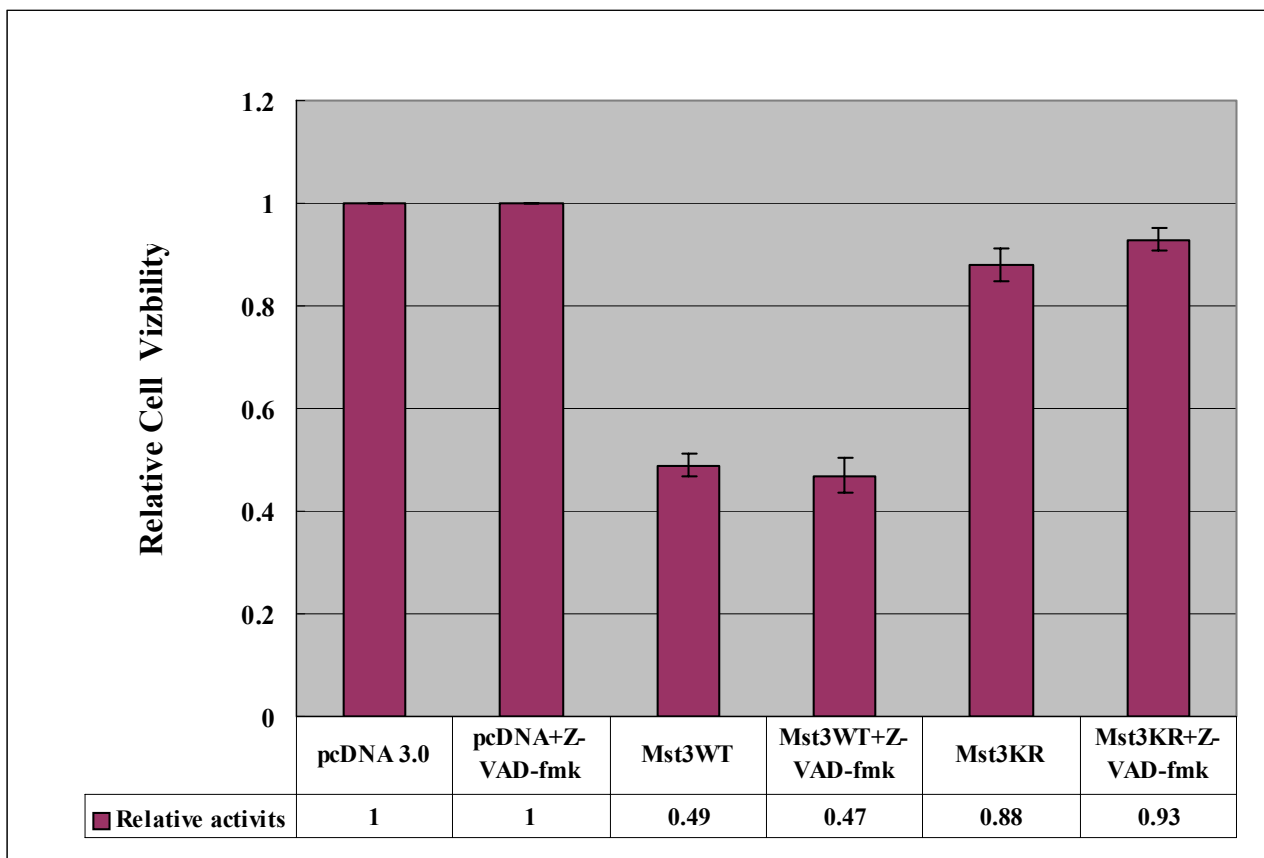
Fig. 2 Effect of the pan-inhibitor Z-VAD-fmk on Mst3 induced apoptosis.
HeLa cells were co-transfected with pSV-LacZ and pcDNA3/Mst3^{WT}or
pcDNA3/Mst3^{KR}plasmids. Six hours after transfection , cells were left untreated or
treated with Z-VAD-fmk (50 μM) at 37℃ for 24 h after treatment, cells harvested
and assay for β-galactosidase activity. Results shown are mean ± S.E of three
independent experiments. The β-galactosidase activity of pcDNA3.0-transfected
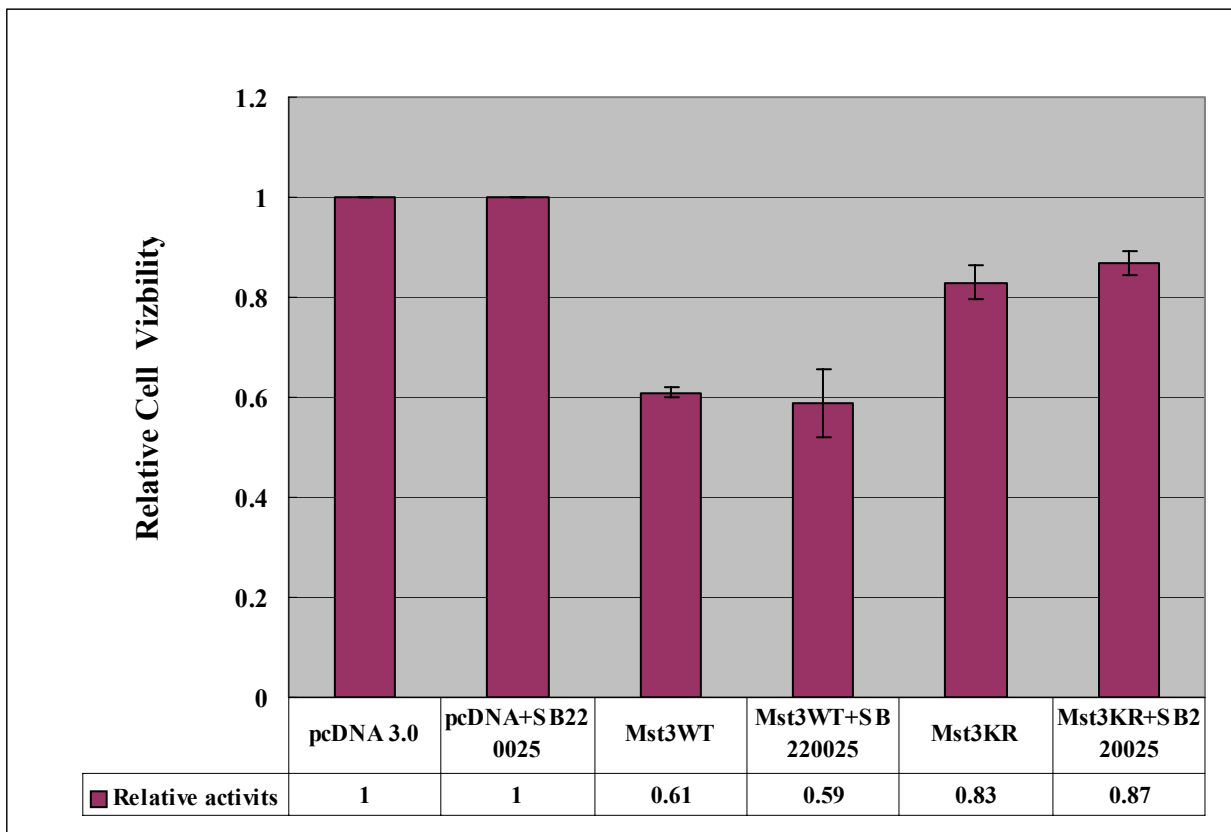cells was used as 1.0.
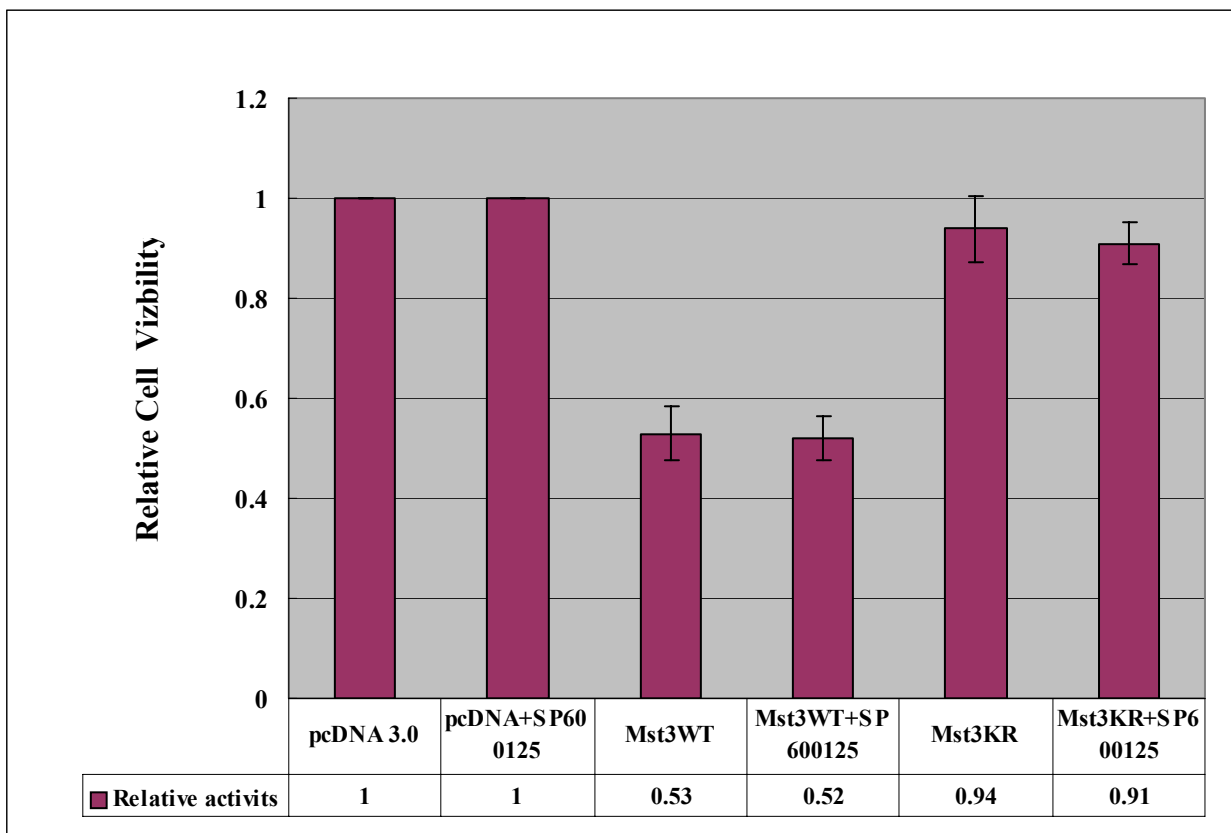
Fig. 3 Effect of the JNK inhibitor (SP600125) on Mst3 induced apoptosis. HeLa cells were co-transfected with pSV-LacZ and pcDNA3/Mst3^{WT} or pcDNA3/Mst3^{KR} vectors. Six hours after transfection, cells were left untreated or treated with JNK inhibitor SP600125 (0.5 μM) at 37℃ for 24 h. After incubation, cells were harvested and assay for β-galactosidase activity. Results shown are mean ± S.D of three independent experiments. The β-galactosidase activity of pcDNA3.0-transfected cells was used as 1.0.

	pcDNA 3.0	pcDNA+SB220025	Mst3WT	Mst3WT+SB220025	Mst3KR	Mst3KR+SB220025
■ Relative activits	1	1	0.61	0.59	0.83	0.87

Fig. 4 Effect of the p38 MAPK inhibitor (SB220025) on Mst3 induced apoptosis. HeLa cells were co-transfected with pSV-LacZ and pcDNA3/Mst3[WT]or pcDNA3/Mst3[KR] vectors. Six hours after transfection, cells were left untreated or treated with p38 MAPK inhibitor SB220025 (0.5 μM) at 37℃ for 24 h. Cells were then harvested and assay for β-galactosidase activity. Results shown are mean ± S.D of three independent experiments. The β-galactosidase activity of pcDNA3.0-transfected cells was used as 1.0.

(A)

(B)
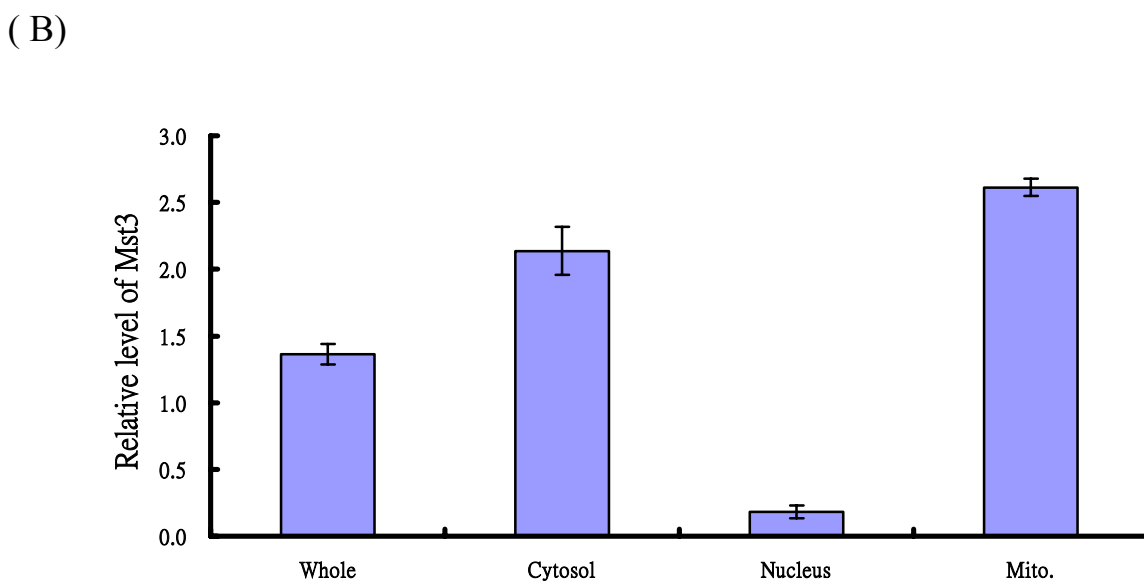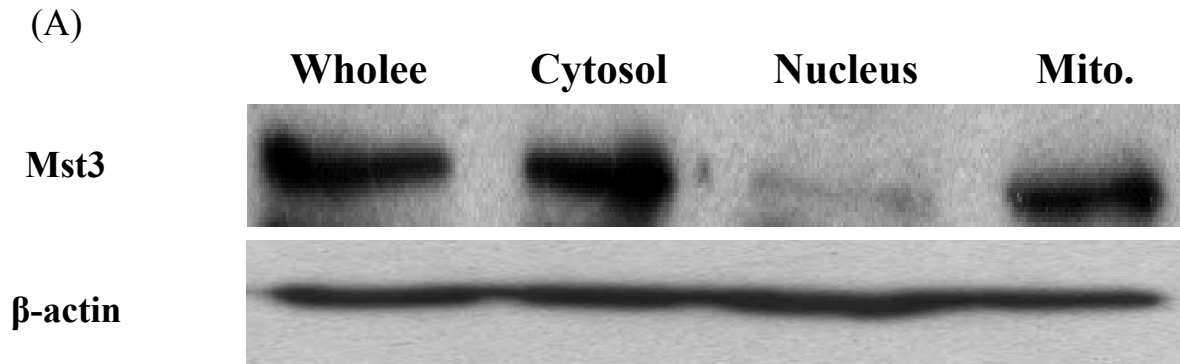
Fig. 5 Localization of endogenous Mst3 in HeLa cell. (A) Western blot analysis of the endogenous Mst3 in HeLa cells, Cells were grown to about 80-90% confluences and then the whole cell lysate, cytoplasmic, nuclear and mitochondrial fractions were isolated as described in Materials and Methods. Sample (60 μg each) was loaded and separated on a 12% SDS-PAGE. β-actin was used as internal control. Mst3 is mainly present in cytoplasm and mitochondria. (B) The relative amount of Mst3 of different subcellular fractions in HeLa cell. The density of β-actin bend in each lane was viewed as 1 to normalize the density of Mst3 in each fraction.

	EGFP	Mitotracker	Merge

(A)
EGFP

(B)
EGFP-
Mst3^WT

(C)
EGFP-
Mst3^KR
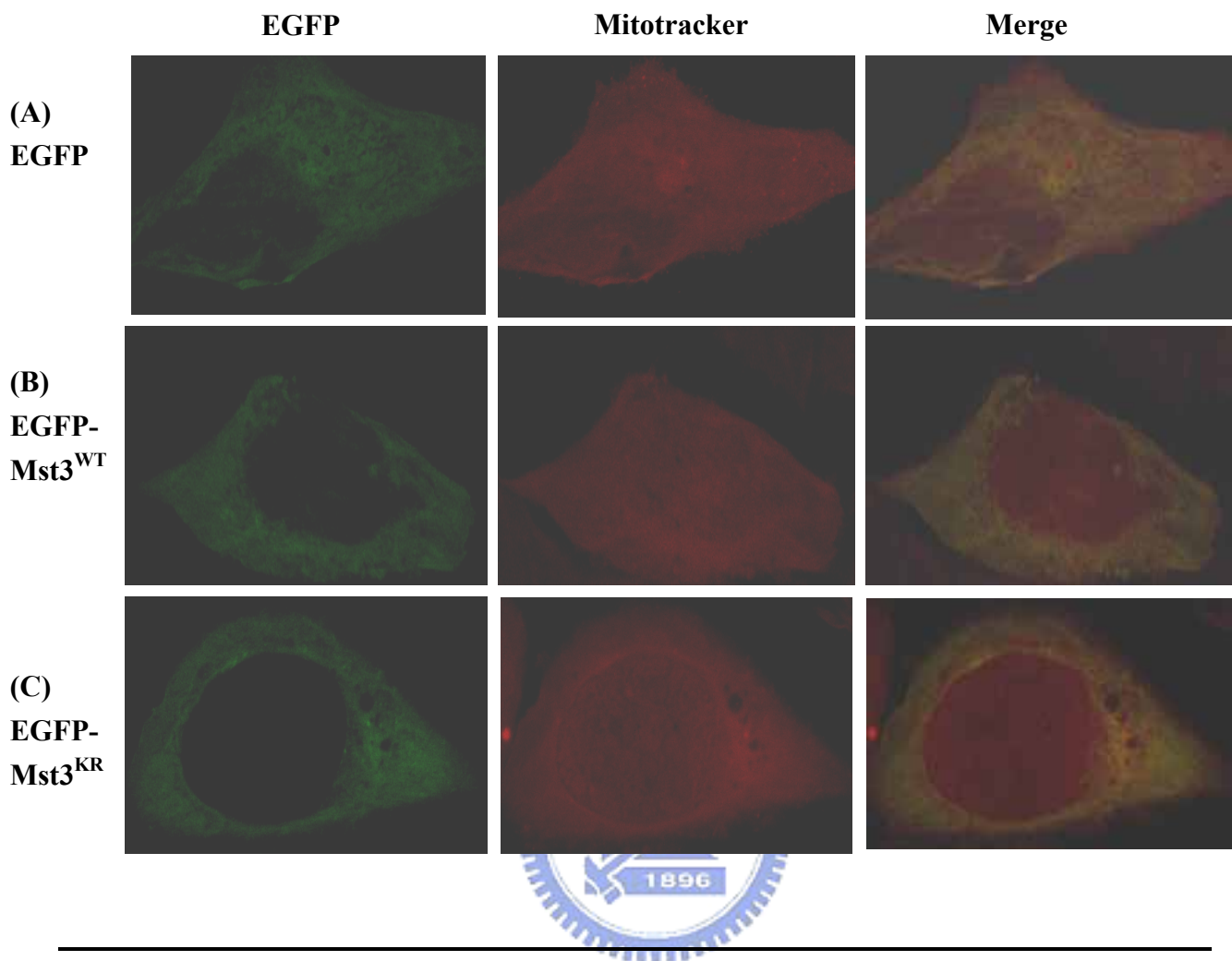
Fig. 6 Localization of EGFP-Mst3 in mitochondria. HeLa cells were transiently transfected with (A) EGFP vector only, (B) EGFP-Mst3^{WT} or (C) EGFP-Mst3^{KR}. Sixteen hours after transfection, cells were fixed and stained with the mitochondrial marker, mitotracker (Molecular Probe). Fluorescent images were monitored under a confocal laser scanning microscope (FV500, OLYMPUS). The yellow color indicates the co-localization of EGFP- Mst3^{WT} (green) and mitotracker (red).

	FITC	Mitotracker	Merge

(A) cyto-chrome C

(B) AIF
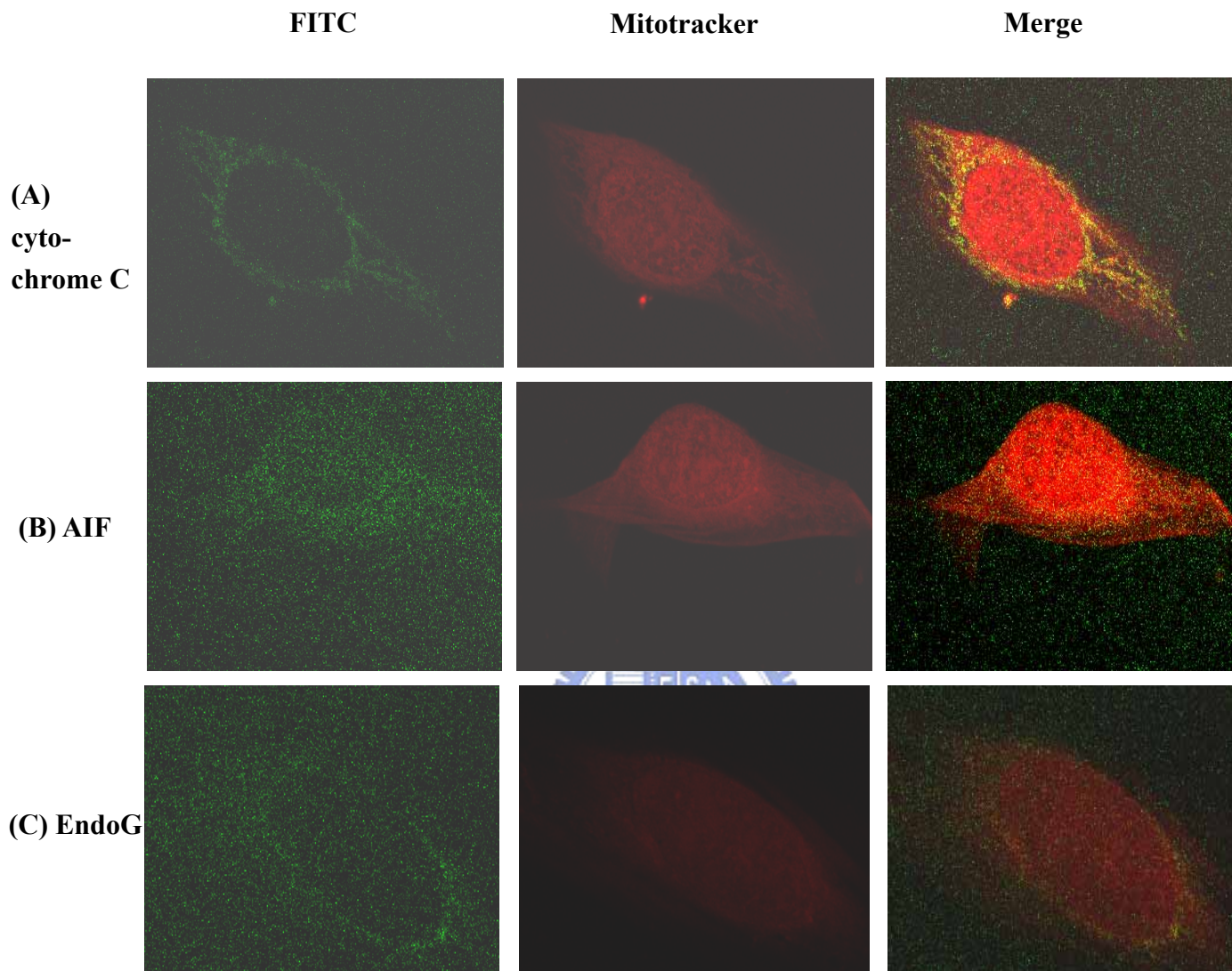
(C) EndoG

Fig. 7 Localization of cytochrome C, AIF and Endo G in mitochondria. The apoptotic factors in HeLa cells were immunofluorescent stained using mouse-anti cytochrome C, mouse-anti AIF and rabbit-anti EndoG antibodies, followed by staining with a FITC-conjugated secondary antibody ageist mouse or rabbit IgG. Mitochondria in HeLa cells were stained with a specific marker, mitotracker red (Molecular Probe). Fluorescent images were observed under a confocal laser scanning microscope (FV500, OLYMPUS). The co-localization of apoptotic factors (green) and mitochondria (red) was visualized as yellow color in the merged images.

	EGFP	**Cytochrome C**	**Merge**

(A) EGFP-Mst3^{WT}
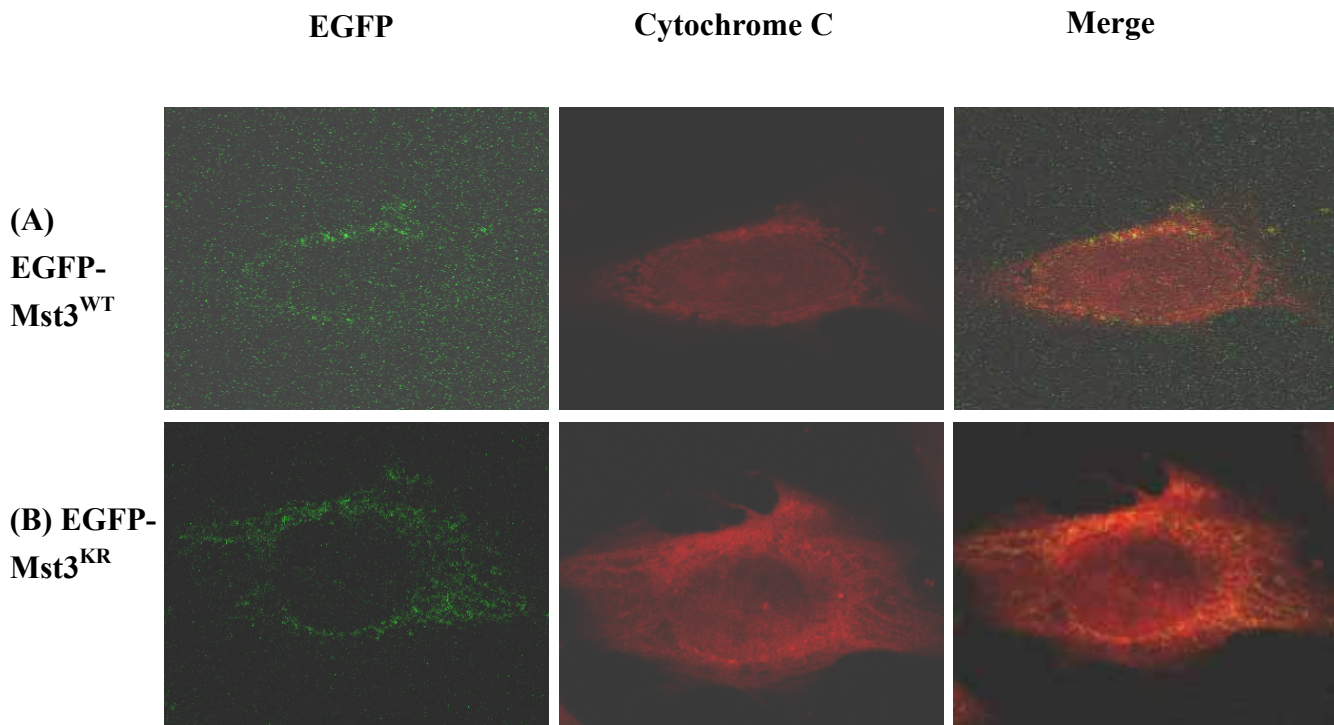
(B) EGFP-Mst3^{KR}

Fig. 8 Co-localization of Mst3 and cytochrome C in mitochondria. HeLa cells were transiently transfected with EGFP-Mst3^{WT} (A) or EGFP-Mst3^{KR} (B). Sixteen hours after transfection, immunofluorescent staining of cytochrome C was performed using mouse anti cytochrome C antibody and visualized by a Rhodamine-conjugated secondary antibody anti-mouse IgG. Fluorescent images were observed under a confocal laser scanning microscope (FV500, OLYMPUS). The co-localization of EGFP-Mst3^{WT} or EGFP-Mst3^{KR} (green color) and cytochrome C (red) was demonstrated as the yellow color in the merged images.
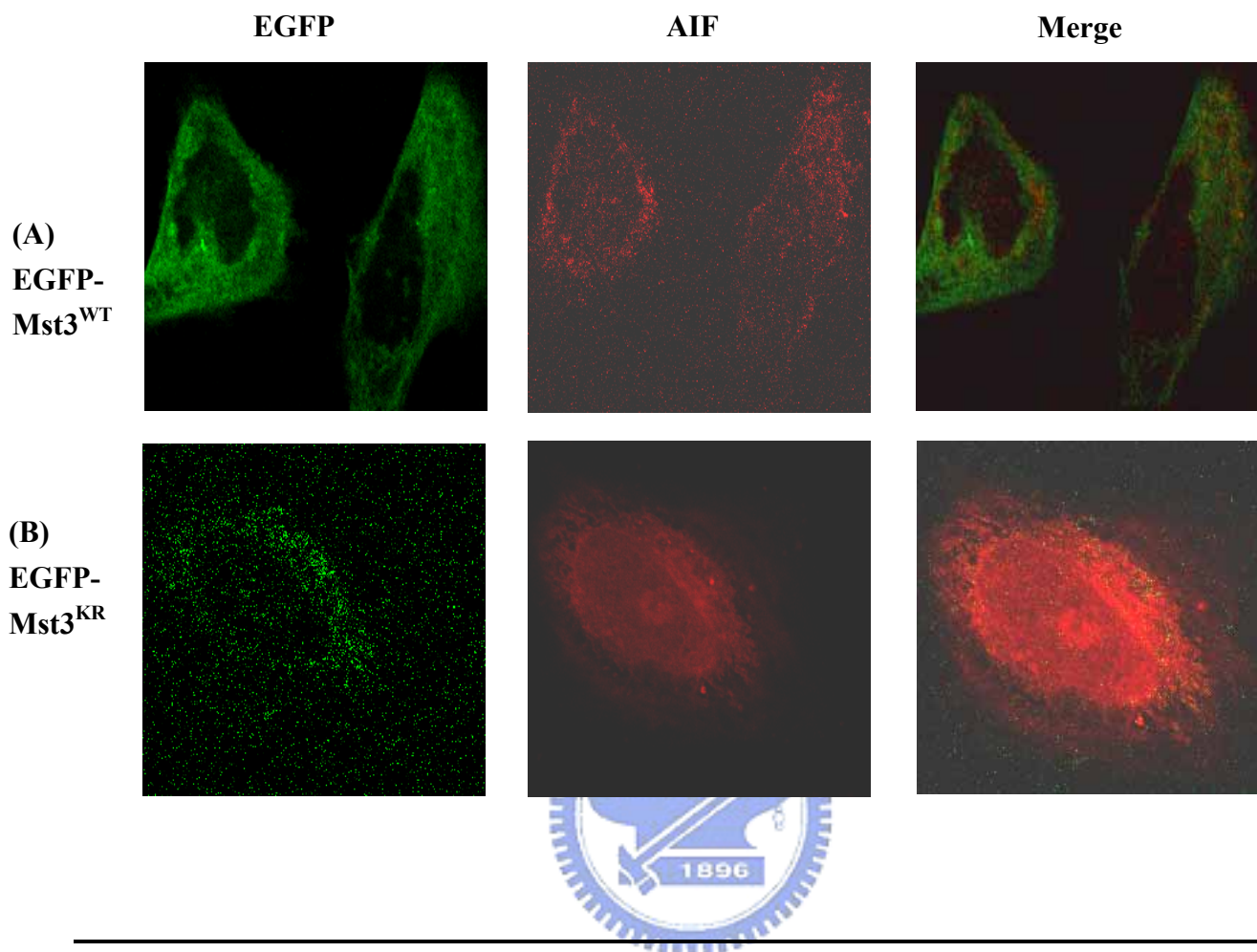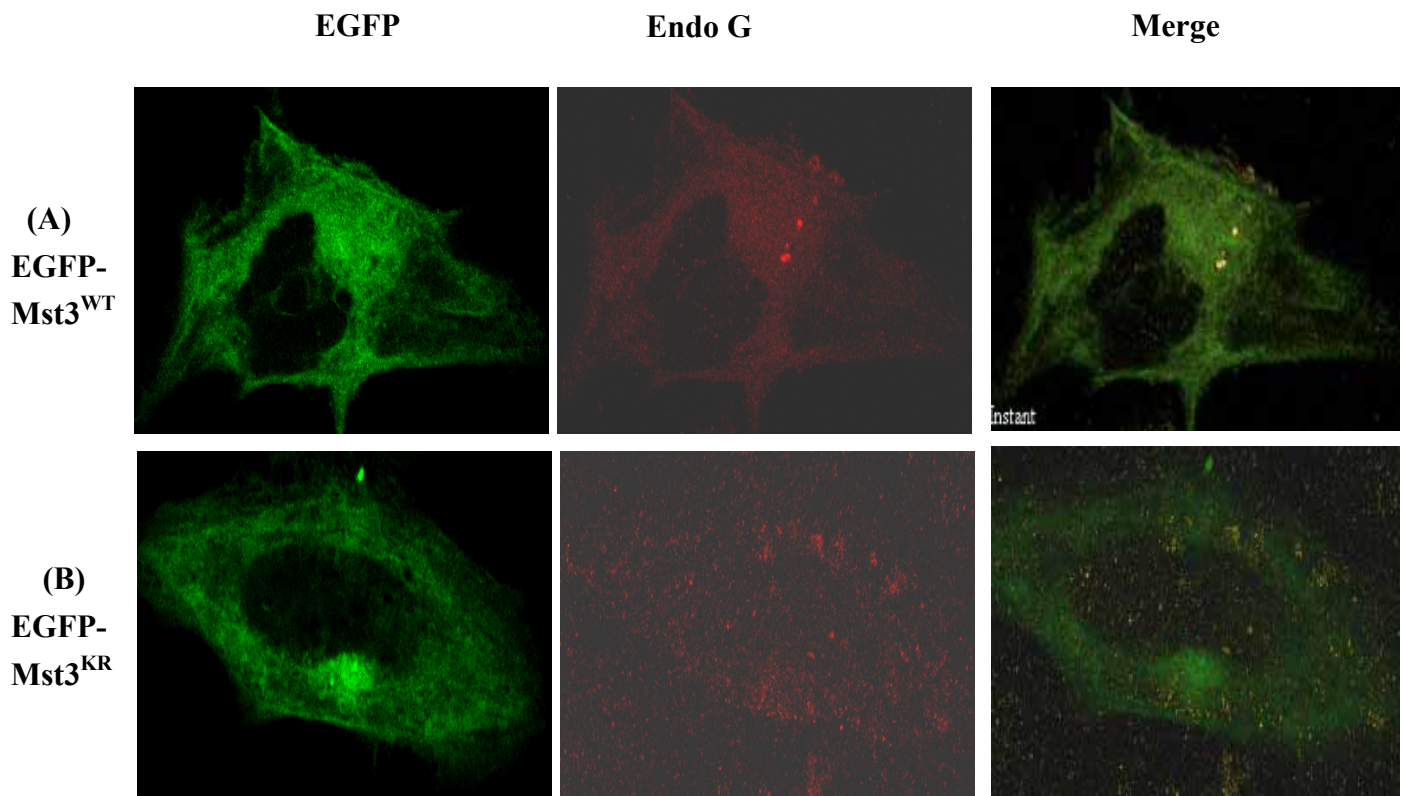
Fig. 9 Immunofluorescent Staining in HeLa cells. HeLa cells were transiently transfected with EGFP-Mst3^{WT} (A) or EGFP-Mst3^{KR} (B). Sixteen hours after transfection, AIF was recognized mouse using anti-AIF antibody and visualized by a Rhodamine-conjugated secondary anti-mouse antibody. Fluorescent images were observed under a confocal laser scanning microscope (FV500, OLYMPUS). The co-localization of EGFP-Mst3^{KR} (green) and AIF (red) was demonstrated as the yellow color in the merged images.

	EGFP	**Endo G**	**Merge**

(A)
EGFP-Mst3^{WT}

(B)
EGFP-Mst3^{KR}

Fig. 10 Immunofluorescent Staining in HeLa cells. HeLa cells were transiently transfected with EGFP-Mst3^{WT} (A) or EGFP-Mst3^{KR} (B). Sixteen hours after transfection, EndoG was recognized using rabbit anti-Endo G antibody and visualized by a Rhodamine-conjugated secondary anti-rabbit antibody. Fluorescent images were observed under a confocal laser scanning microscope (FV500, OLYMPUS).
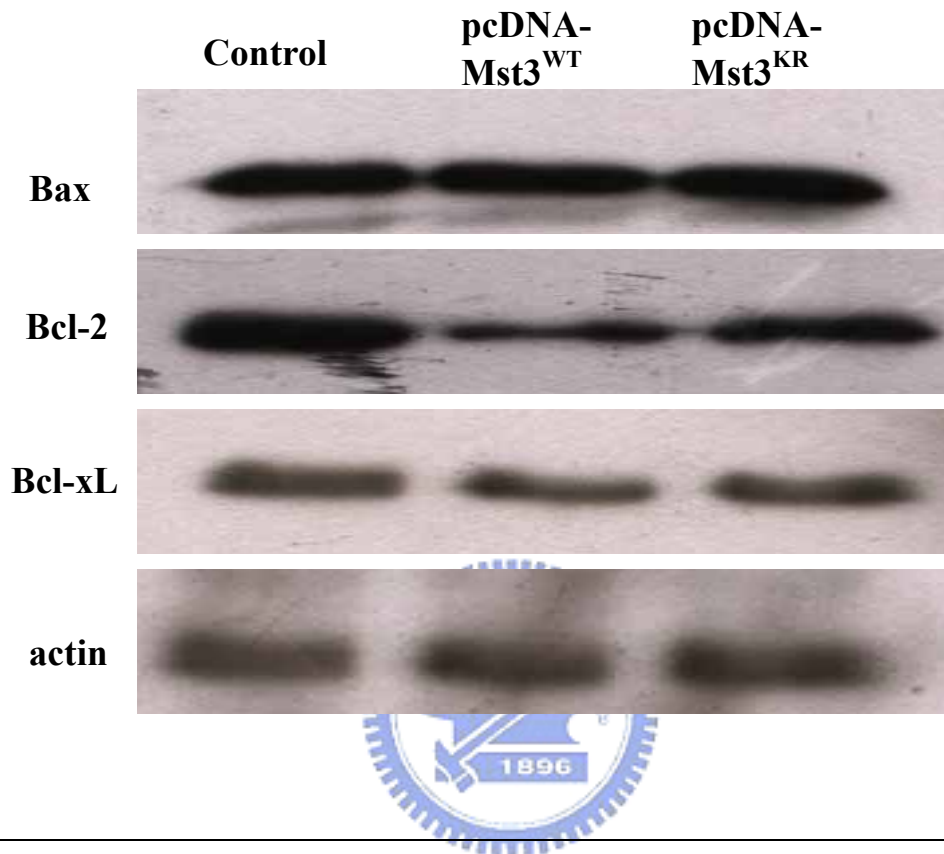
Fig. 11 Expression level of Bcl-2 in the presence of Mst3^{WT} and Mst3^{KR}. Whole cell extracts (50 μg**)** from pcDAN-Mst3^{WT} and pcDAN-Mst3^{KR}-transfected HeLa cells were separated on a 12% SDS-PAGE, followed by probing the protein bends of Bax, Bcl-2 and Bcl-xL using specific antibodies. Actin was used as internal control.

(A)

	Whole	**Cytosol**	**Nucleus**	**Mito.**

Bcl-2
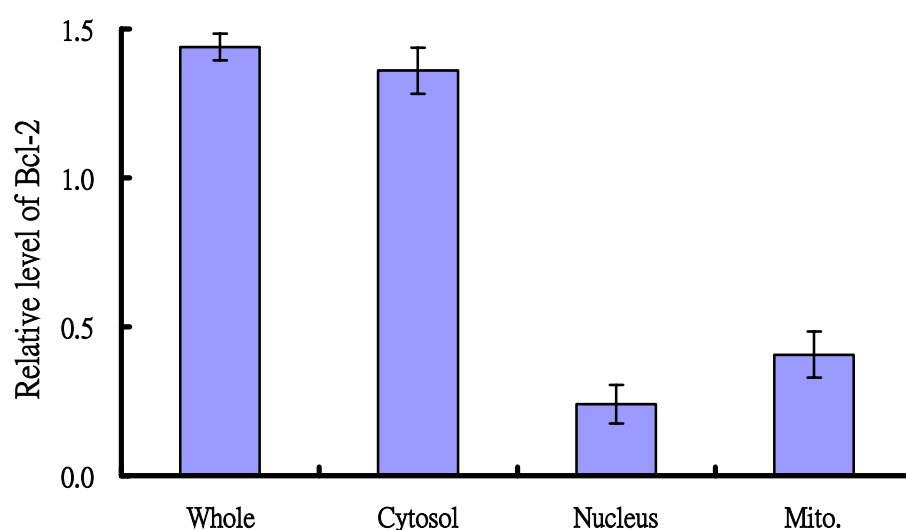
β-actin

(B)

Relative level of Bcl-2
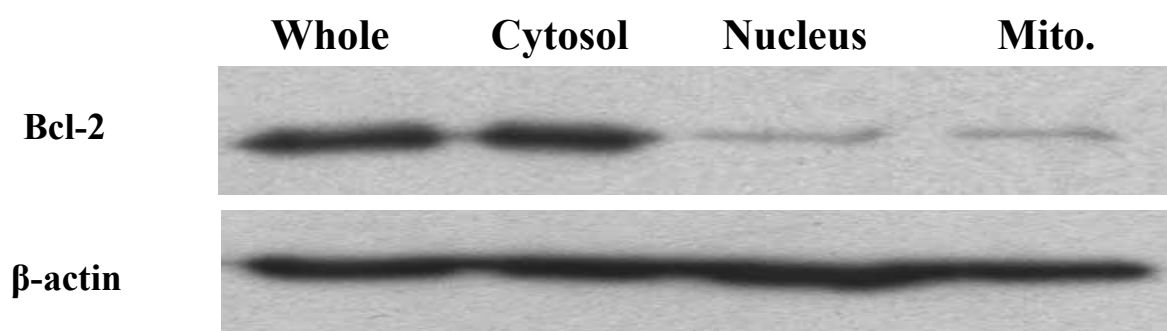
Fig. 12   Subcellular distribution of Bcl-2 in HeLa cells. Endogenous Bcl-2 was determined in whole cell extract, cytosolic fraction, nuclear fraction and mitochondrial fractions by Western blotting. Samples (60 μg each) various fractions samples were separated on a 12% SDS-PAGE, followed by the western blotting analysis. (B) The relative amount of Bcl-2 of different cell fractions of HeLa cell. The density of β-actin in each lane was viewed as 1 to normalize the density of Mst3 in each fraction.
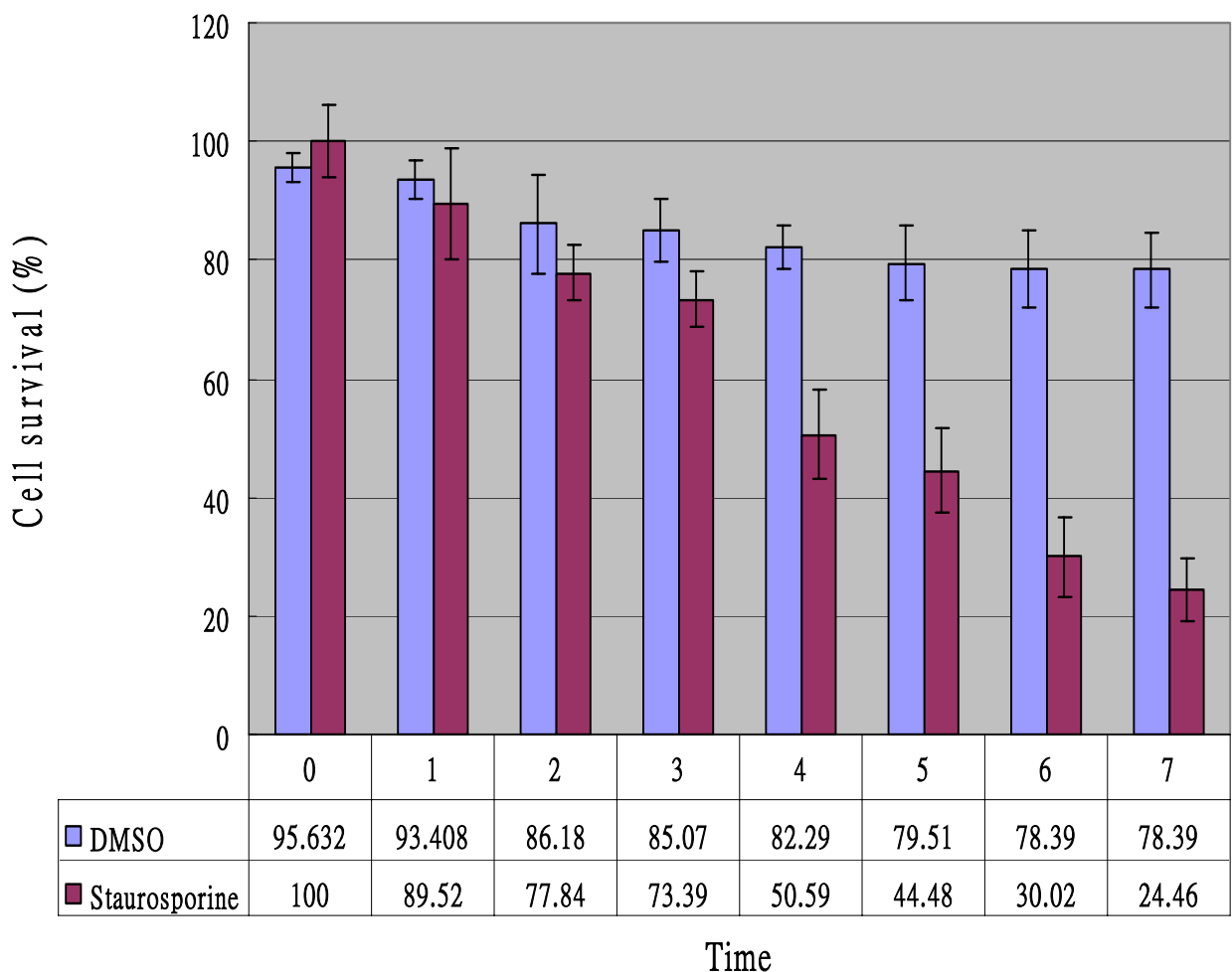
	0	1	2	3	4	5	6	7
☐ DMSO	95.632	93.408	86.18	85.07	82.29	79.51	78.39	78.39
◼ Staurosporine	100	89.52	77.84	73.39	50.59	44.48	30.02	24.46

Fig. 13 Time-dependent effect of sturosporine on HeLa cells. HeLa cells treated with DMSO or 0.5 μM staurosporine at 37℃ for different time. The cells were harvested and assay for MTT activity Assy. Results shown are mean ± S.D of three independent experiments.
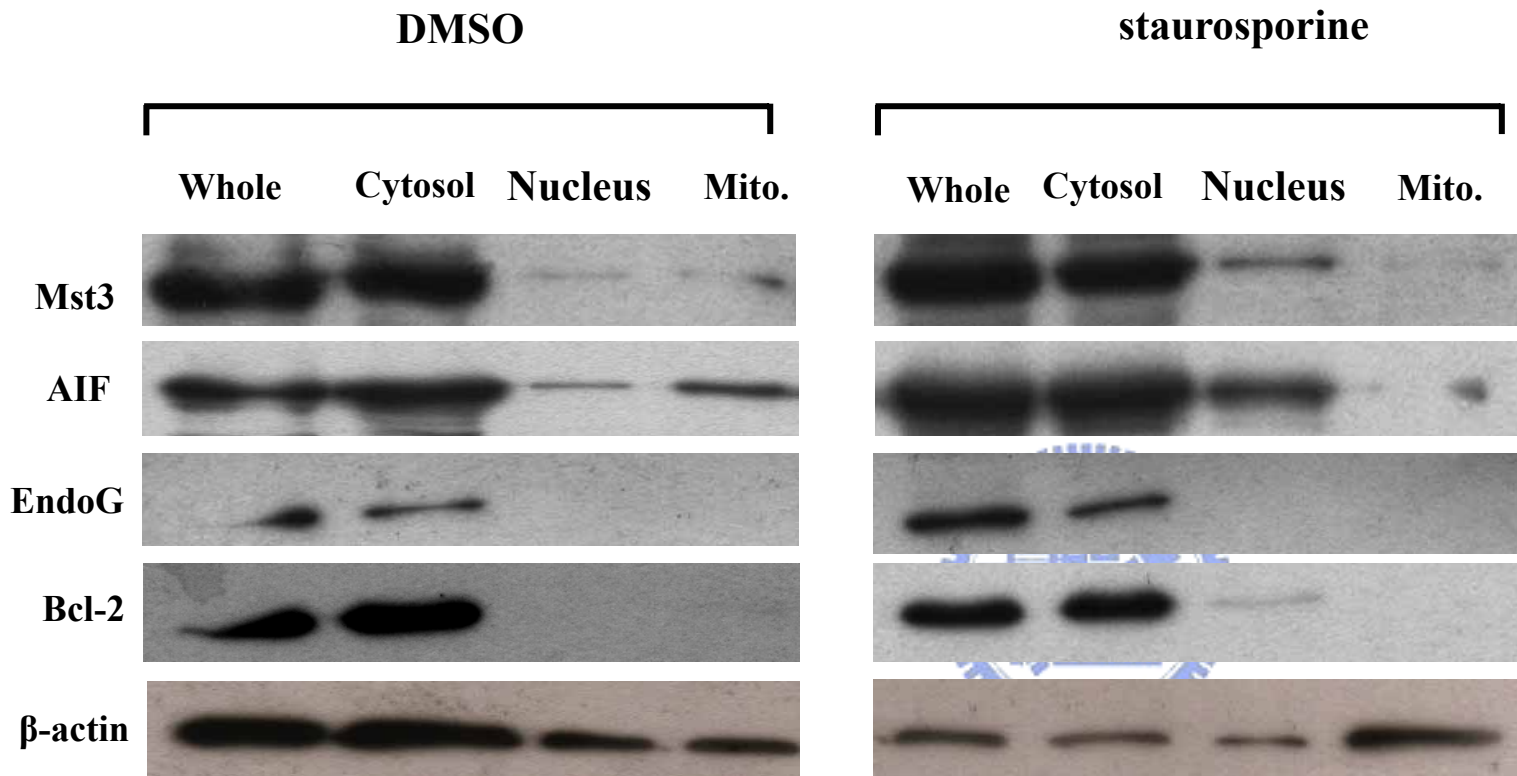
Fig. 14 The effect of staurosporine on the subcellular dietribution of Mst3 and apoptotic regulation factors in HeLa cell. HeLa cells treated with DMSO or 0.5 μM staurosporine at 37℃ for 3 hours were fractionated to isolated the whole protein, cytoplasmic, nuclear and mitochondrial fractions Various sample (40 μg) were separated on a 12% SDS-PAGE. The resulting protein bands were then transferred to PVDF membrane and subjected to Western blotting. Antibodies against Mst3, AIF, EndoG, Bcl-2 and β-actin were used to detected the coverpouding proteins on the PVDF membr
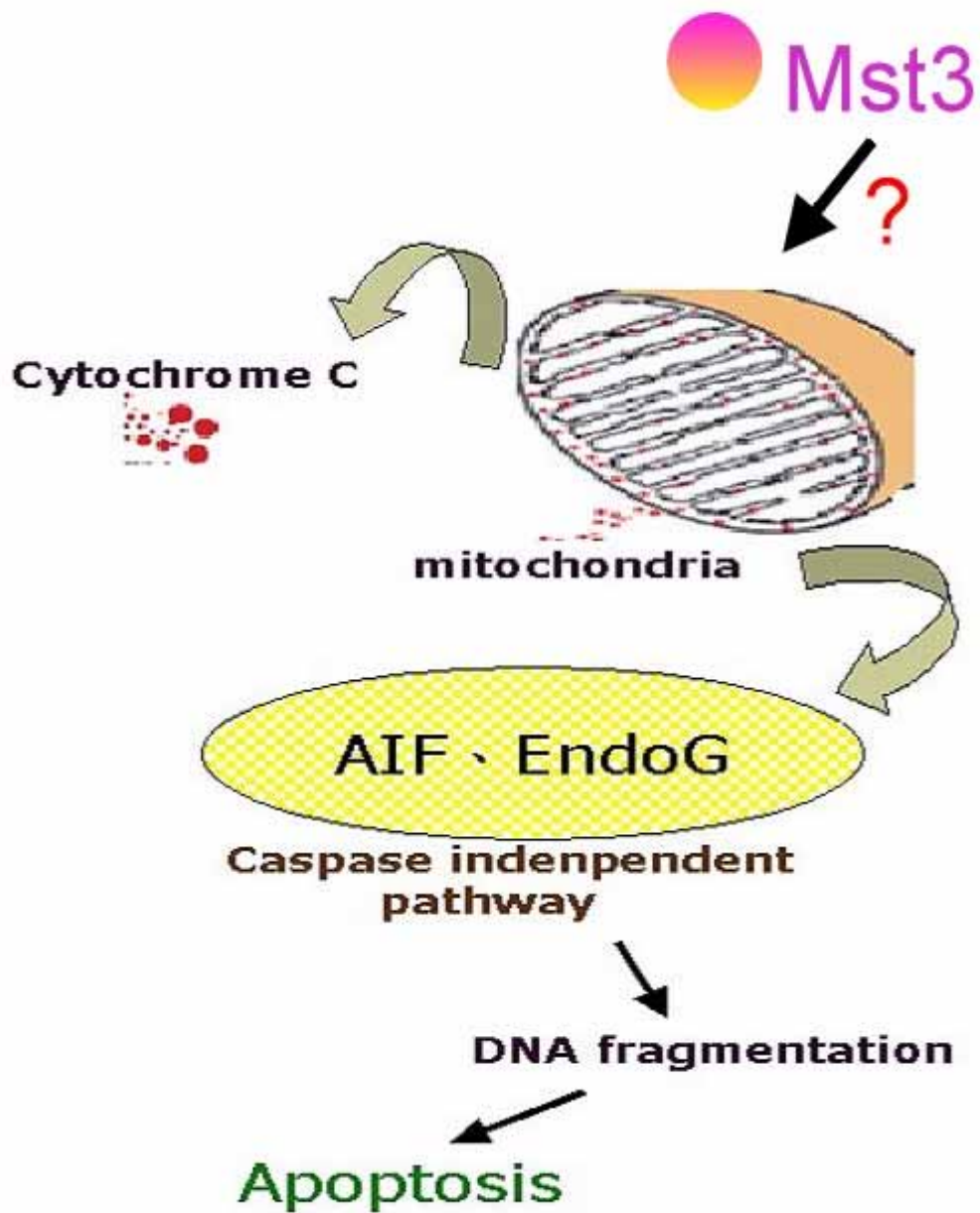
Apoptosis pathway
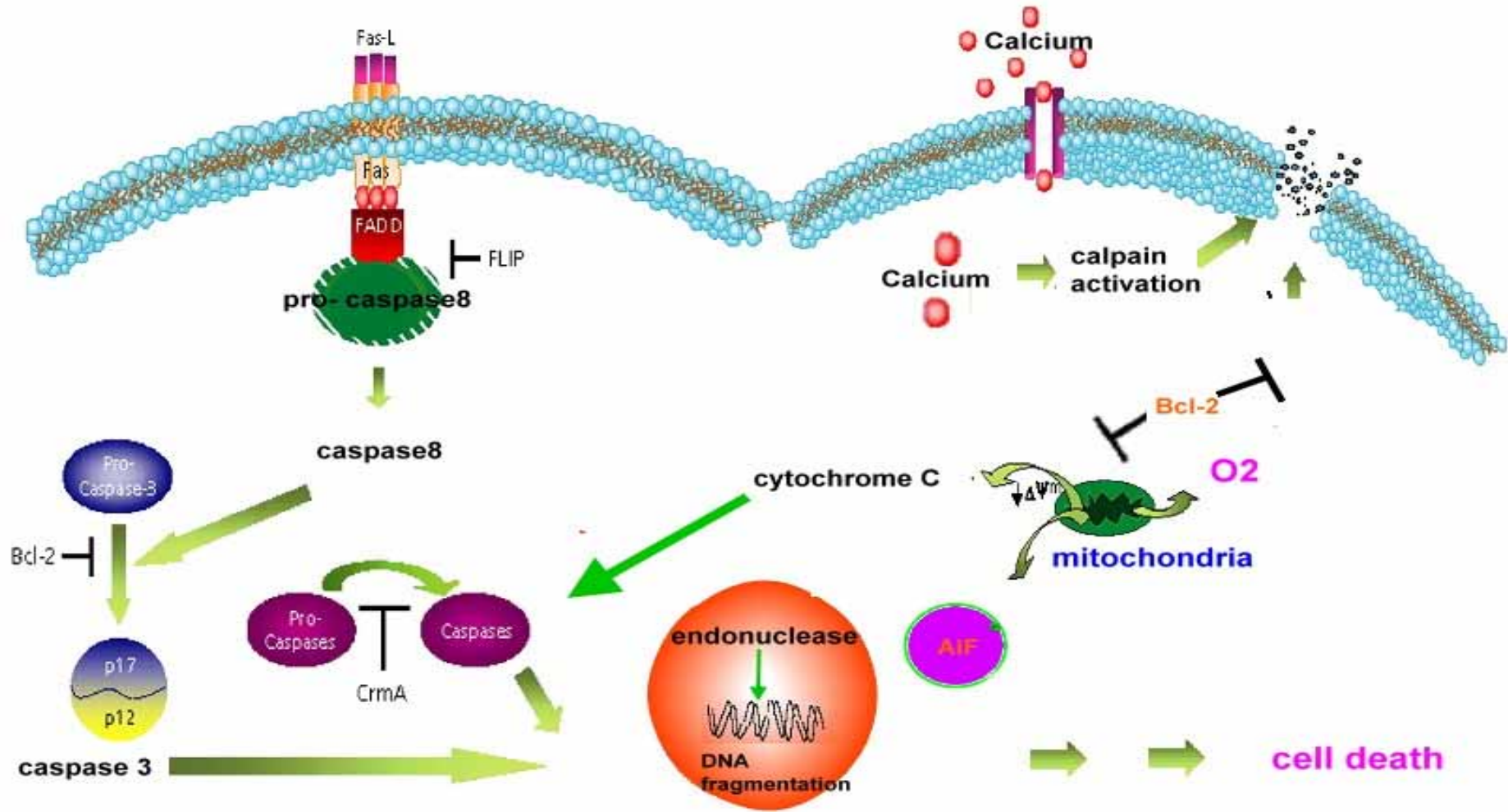
Proposed pathway in Jurkat cells
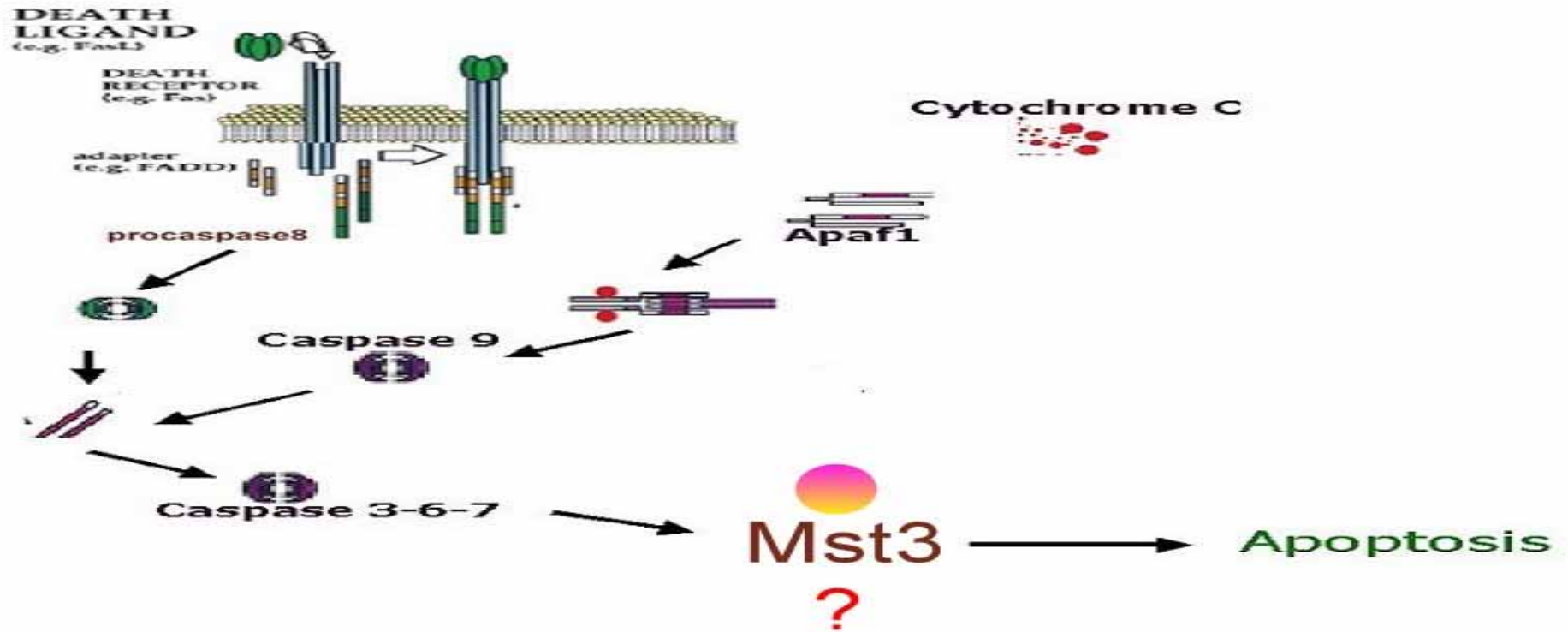
Proposed pathway in HeLa cells

Appendix I

## Nucleotide and amino acid sequence of Mst3.

```
GGCCCGCGGGCCTCGCCGCCCCGCGCGGATCGTCGCGGCCCGGCCGTCCCGTCCCAGGAAGTGGCCGTCCTGAGCGCCATGGCT      90
                                                                               M  A      2

CACTCCCCGGTGCAGTCGGGCCTGCCCGGCATGCAGAACCTAAAGGCAGACCCAGAAGAGCTTTTTACAAAACTAGAGAAAATTGGGAAG   180
 H  S  P  V  Q  S  G  L  P  G  M  Q  N  L  K  A  D  P  E  E  L  F  T  K  L  E  K  I  G  K     32

GGCTCCTTTGGAGAGGTGTTCAAAGGCATTGACAATCGGACTCAGAAAGTGGTTGCCATAAAGATCATTGATCTGGAAGAAGCTGAAGAT   270
 G  S  F  G  E  V  F  K  G  I  D  N  R  T  Q  K  V  V  A  I  K  I  I  D  L  E  E  A  E  D     62

GAGATAGAGGACATTCAACAAGAAATCACAGTGCTGAGTCAGTGTGACAGTCCATATGTAACCAAATATTATGGATCCTATCTGAAGGAT   360
 E  I  E  D  I  Q  Q  E  I  T  V  L  S  Q  C  D  S  P  Y  V  T  K  Y  Y  G  S  Y  L  K  D     92

ACAAAATTATGGATAATAATGGAATATCTTGGTGGAGGCTCCGCACTAGATCTATTAGAACCTGGCCCATTAGATGAAACCCAGATCGCT   450
 T  K  L  W  I  I  M  E  Y  L  G  G  G  S  A  L  D  L  L  E  P  G  P  L  D  E  T  Q  I  A    122

ACTATATTAAGAGAAATACTGAAAGGACTCGATTATCTCCATTCGGAGAAGAAAATCCACAGAGACATTAAAGCGGCCAACGTCCTGCTG   540
 T  I  L  R  E  I  L  K  G  L  D  Y  L  H  S  E  K  K  I  H  R  D  I  K  A  A  N  V  L  L    152

TCTGAGCATGGCGAGGTGAAGCTGGCGGACTTTGGCGTGGCTGGCCAGCTGACAGACACCCAGATCAAAAGGAACACCTTCGTGGGCACC   630
 S  E  H  G  E  V  K  L  A  D  F  G  V  A  G  Q  L  T  D  T  Q  I  K  R  N  T  F  V  G  T    182

CCATTCTGGATGGCACCCGAGGTCATCAAACAGTCGGCCTATGACTCGAAGGCAGACATCTGGTCCCTGGGCATAACAGCTATTGAACTT   720
 P  F  W  M  A  P  E  V  I  K  Q  S  A  Y  D  S  K  A  D  I  W  S  L  G  I  T  A  I  E  L    212

GCAAGAGGGGAACCACCTCATTCCGAGCTGCACCCCATGAAAGTTTTATTCCTCATTCCAAAGAACAACCCACCGACGTTGGAAGGAAAC   810
 A  R  G  E  P  P  H  S  E  L  H  P  M  K  V  L  F  L  I  P  K  N  N  P  P  T  L  E  G  N    242

TACAGTAAACCSCTCAAGGAGTTTGTGGAGGCCTGTTTGAATAAGGAGCCGAGCTTTAGACCCACTGCTAAGGAGTTATTGAAGCACAAG   900
 Y  S  K  P  L  K  E  F  V  E  A  C  L  N  K  E  P  S  F  R  P  T  A  K  E  L  L  K  H  K    272

TTTATACTACGCAATGCAAAGAAAACTTCCTACTTGACCGAGCTCATCGACAGGTACAAGAGATGGAAGGCCGAGCAGAGCCATGACGAC   990
 F  I  L  R  N  A  K  K  T  S  Y  L  T  E  L  I  D  R  Y  K  R  W  K  A  E  Q  S  H  D  D    302

TCGAGCTCCGAGGATTCCGACGCGGAAACAGATGGCCAAGCCTCGGGGGGGCAGTGATTCTGGGGACTGGATCTTCACAATCCGAGAAAAA  1080
 S  S  S  E  D  S  D  A  E  T  D  G  Q  A  S  G  G  S  D  S  G  D  W  I  F  T  I  R  E  K    332

GATCCCAAGAATCTCGAGAATGGAGCTCTTCAGCCATCGGACTTGGACAGAAATAAGATGAAAGACATCCCAAAGAGGCCTTTCTCTCAG  1170
 D  P  K  N  L  E  N  G  A  L  Q  P  S  D  L  D  R  N  K  M  K  D  I  P  K  R  P  F  S  Q    362

TGTTTATCTACAATTATTTCTCCTCTGTTTGCAGAGTTGAAGGAGAAGAGCCAGGCGTGCGGAGGGAACTTGGGTTCCATTGAAGAGCTG  1260
 C  L  S  T  I  I  S  P  L  F  A  E  L  K  E  K  S  Q  A  C  G  G  N  L  G  S  I  E  E  L    392

CGAGGGGCCATCTACCTAGCGGAGGAGGTGTGCCCTGGCATCTCCGACACCATGGTGGCCCAGCTCGTGCAGCGGCTCCAGAGATACTCT  1350
 R  G  A  I  Y  L  A  E  E  V  C  P  G  I  S  D  T  M  V  A  Q  L  V  Q  R  L  Q  R  Y  S    422

CTAAGTGGTGGAGGAACTTCATCCCACTGAAATTCCTTTGGCATTTGGGGTTTTGTTTTTCCTTTTTTCCTTCTTCATCCTCCTCCTTTT  1440
 L  S  G  G  G  T  S  S  H  •                                                                431

TTAAAAGTCAACGAGAGCCTTCGCTGACTCCACCGAAGAGGTGCGCCACTGGGAGCCACCCCAGCSCCAGGCGCCCGTCCAGGGACACAC  1530
ACAGTCTTCGCTGTGCTGCAGCCAGATGAAGTCTCTCAGATGGGTGGGGAGGGTCAGCTCCTTCCAGCGATCATTTTATTTTATTTTATT  1620
ACKTTTGTTTTTAATTTTAACCATAGCGCACATATTCCAGGAAAGTGTCTTTAAAAACAAAAACAAACCCTGAAATGTATATTTGGGATT  1710
ATGATAAGGCAACTAAAGACATGAAACCTCAGGTATCCTGCTTTAAGTTGATAACTCCCTCTGGGAGCTGGAGAATCGCTCTGGTGGATG  1800
GGTGTACAGATTTGTATATAATGTCATTTTTACGGAAACCCTTTCGGCGTGCATAAGGAATCACTGTGTACAAACTGGCCAAGTGCTTCT  1890
GTAGATAACGTCAGTGGAGTAAATATTCGACAGGCCATAACTTGAGTCTATTGCCTTGCCTTTATTACATGTACATTTTGAATTCC       1976
```

The putative start codon is indicated in bold. The kinase domain is underlined. Numbering of nucleotides and amino acids is indicated on the right.

# Appendix II

The plasmid map of pEGFP-C2 vector
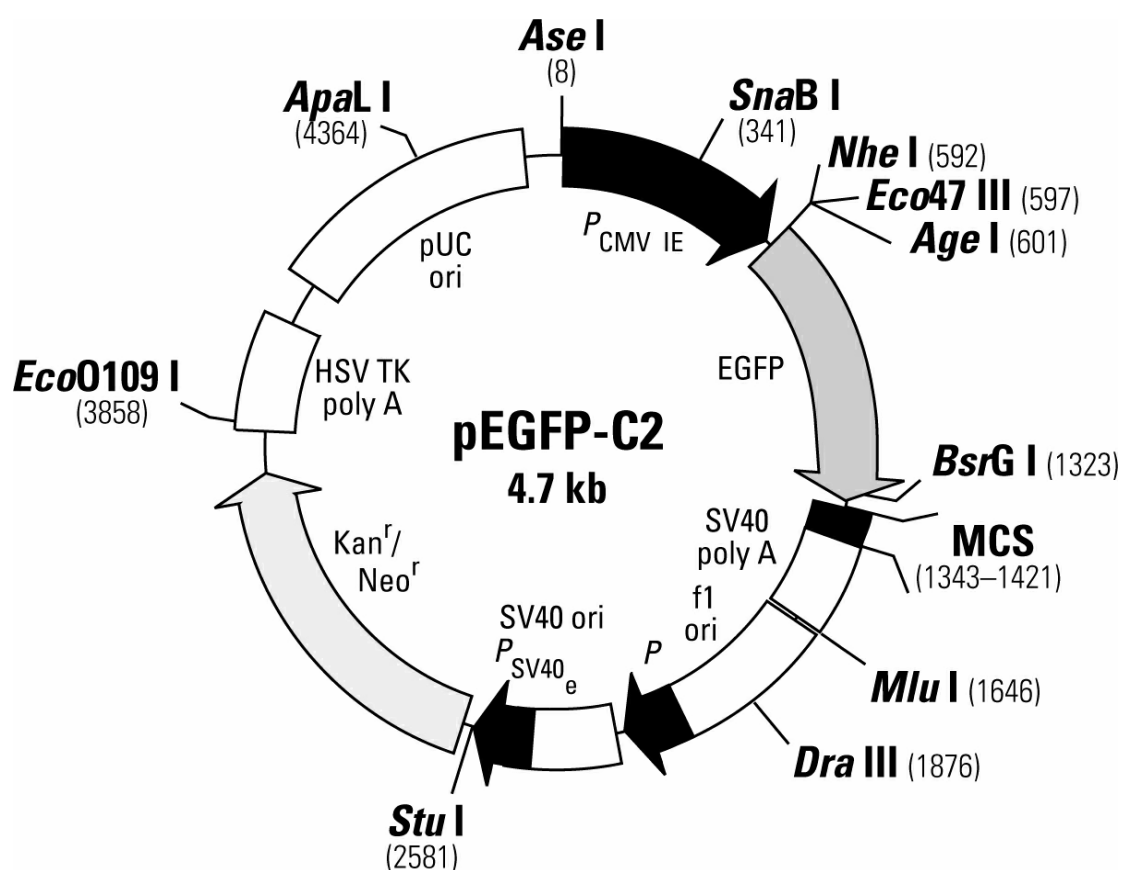
Restriction Map and Multiple Cloning Site (MCS) of pEGFP-C2. (Unique restriction sites are in bold). The *Eag*I site is not unique. The *Xba*I site cannot be used for fusions since it contains an in-frame stop codon. The *Xba*I and *Bcl* I sites(*) are methylated in the DNA provided by CLONTECH. If you wish to digest the vector with these enzymes, you will need to transform the vector into a *dam⁻* host and make fresh DNA.

Appendix III

The plasmid map of pcDNA3 vector
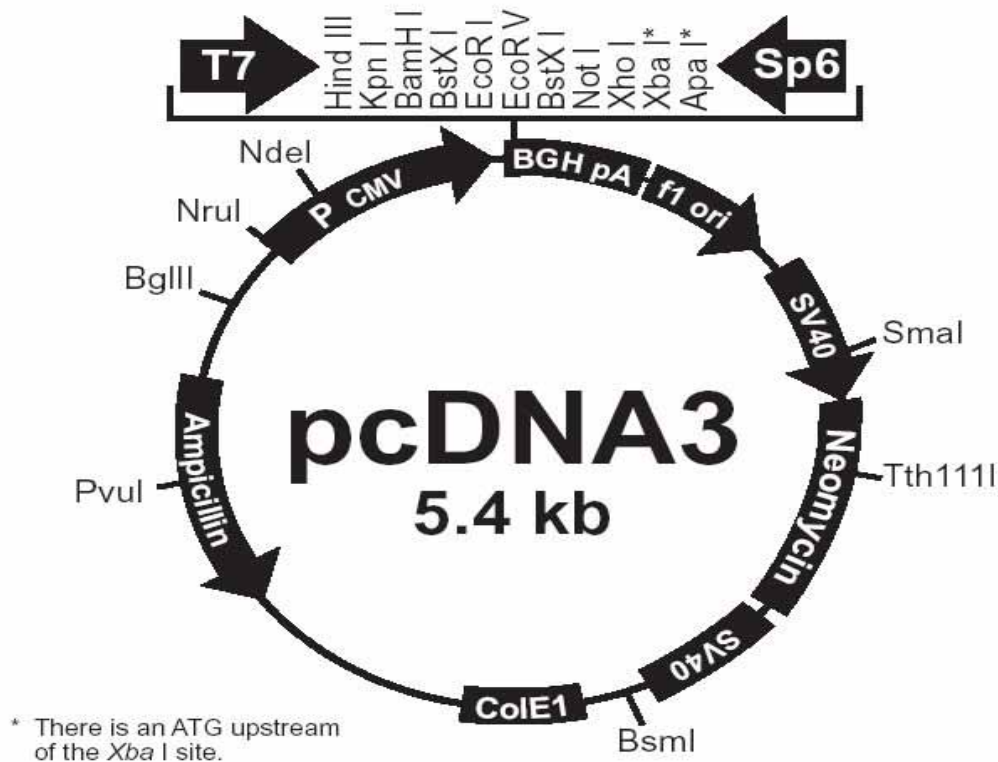
CTC TCT GGC TAA CTA GAG AAC CCA CTG CTT ACT GGC TTA TCG AAA TTA

HA tag -->

ATA CGA CTC ACT ATA GGG AGA CCC <u>AAG CTT</u> <u>GGT ACC</u> ATG GCT TAC CCT

HindIII   *KpnI*

NcoI

TAT GAT GTG CCA GAT TAT GCC T<u>CC CGG GA</u>T CCA CTA GTA ACG GCC GCC

SmaI

BamHI

AGT GTG CTG <u>GAA TTC</u> TGC A<u>GA TAT CC</u>A TCA CAC TGG <u>CGG CCG</u> <u>CTC GAG</u>

EcoRI   *EcoRV*   *NotI*   *XhoI*

CAT GCA <u>TCT AGA</u> <u>GGG CCC</u> TAT TCT ATA GTG TCA CCT AAA TGC TAG AGC
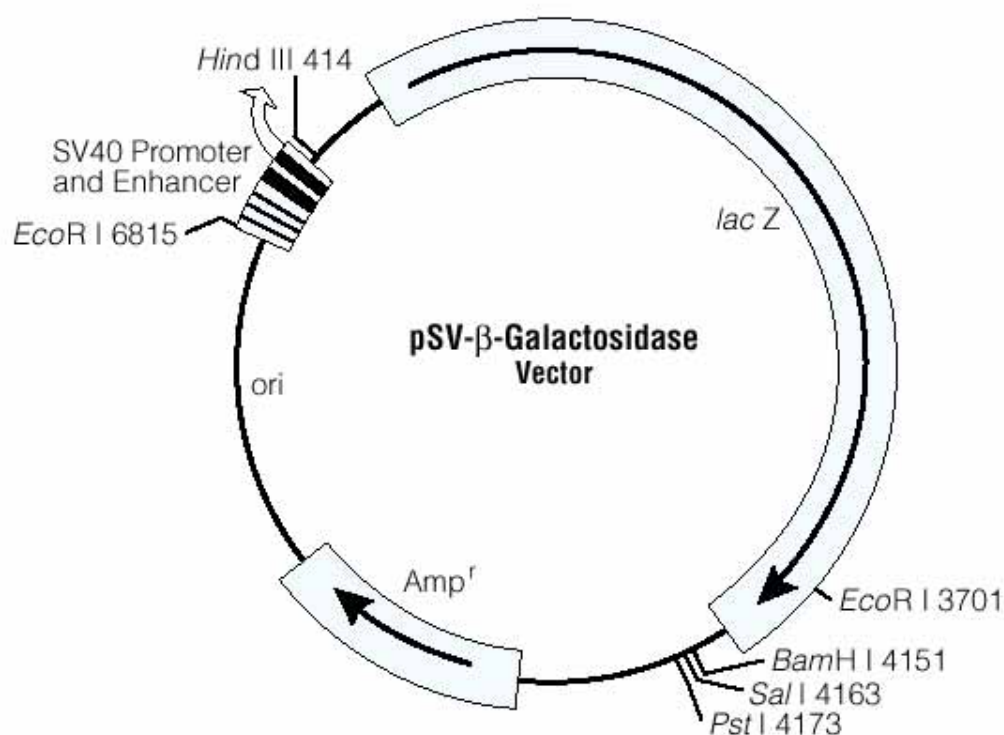
XbaI   *ApaI*

TCG CTG ATC AGC CTC GAC TGT GCC TTC TAG TTG CCA GCC ATC TGT TG

Appendix IV

The plasmid map pSV-β-Galactosidase Vector

Restriction Map of pSV-β-Galactosidase Vector.

The SV40 early promoter and enhancer drive transcription of the bacterial *lacZ* gene, which in turn, is translated into the β-galactosidase enzyme. β-galactosidase is an excellent reporter enzyme, that can be assayed quickly and directly in cell extracts using spectrophotometric, fluorescent or chemiluminescent assays.